THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Biobased Adipic Acid

Challenges in Establishing a Cell Factory

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Cover: Illustration of challenges to adress in order to establish a cell factory for biobased adipic acid production.

"The best way to predict
your future is to create it"

-Peter Drucker

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ABSTRACT

Growing concern regarding climate change calls for sustainable solutions to significantly reduce our dependency on non-renewable fossil-based raw materials. One potential solution is the development of biorefineries where biobased, renewable raw materials are converted into valuable products via enzymatic, chemical or microbial conversion. This work focuses on the microbial production of adipic acid, a precursor in the nylon industry, currently derived from fossil-based raw material. No known naturally occurring microorganism is able to produce adipic acid, and genetic engineering of a suitable host is therefore required.

The aim of the work presented in this thesis was to engineer a microorganism for the production of adipic acid from glucose, more specifically, from glucose streams derived from lignocellulosic forest residues. Theoretical evaluation of metabolic pathways for adipic acid production revealed several obstacles, including redox imbalance and the discovery or engineering of enzymes to catalyze novel reactions. Mining of enzyme databases for alternative paths proved fruitful, and the number of biochemical reactions in the lysine pathway employing as yet unidentified enzymes was reduced from three to two, without affecting the thermodynamics of the pathway. A combined approach of *in vitro* and *in silico* analysis suggested potential enzyme engineering strategies for one of the reactions, for which there are as yet no identified enzymes, namely, the reduction of unsaturated α,β bonds of 6-aminohex-2-enoic acid and 2-hexenedioic acid.

When defining a suitable host for microbial adipic acid production, tolerance to high concentrations of adipic acid (50-100 g L^{-1}) is important to ensure an economically feasible process, preferably at low pH (below 5) to further reduce the overall process cost. Screening of bacteria, yeasts and a filamentous fungus grown in increasing concentrations of adipic acid (0-100 g L^{-1}) and at different pH revealed *Candida viswanathii* to be a promising host to engineer for adipic acid production. A comparative study of *C. viswanathii* with *Saccharomyces cerevisiae* in controlled batch cultivations at increasing adipic acid concentrations (0-95 g L^{-1}) and low pH (pH 4 and pH 5) revealed significant differences in their tolerance to adipic acid; *C. viswanathii* being able to grow, almost unaffected, under all the conditions investigated, whereas *S. cerevisiae* was unable to grow at 95 g L^{-1}. Lipid analysis of their cell membranes revealed *C. viswanathii* to have a thicker and more compact cell membrane, which is probably less permeable to adipic acid.

Keywords: adipic acid, biorefinery, lignocellulose, cell factory, adipic acid pathway via lysine, enzyme engineering, *in silico* docking, 6-aminohex-2-enoic acid, 2-hexenedioic acid, *S. cerevisiae*, *C. viswanathii,* tolerance

Preface

This doctoral thesis fulfils the requirements for a PhD degree at the Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Sweden. The work presented in this thesis was performed between 2014 and 2019, and was funded by the Swedish Research Council for Sustainable Development, "Formas", (Biobased Bulk and Fine Chemicals, no. 2013- 78) under the Programme for strong research environments in bioeconomy.

The aim of this work was to develop a microorganism for adipic acid production from lignocellulosic raw materials. The main part of the work was carried out at the Division of Industrial Biotechnology at Chalmers University of Technology, under the supervision of Professor Lisbeth Olsson and Dr. Valeria Mapelli.

Chemical synthesis of 2-hexenedioic acid and NMR analysis of 6-aminohex-2-enoic acid were performed by Professor Gunnar Westman at the Department of Chemistry and Chemical Engineering, Chalmers University of Technology, Gothenburg, Sweden. The *in silico* studies on the enzymes NemA and Oye1 were performed in collaboration with Professor Leif Eriksson, Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden.

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Emma Skoog
May, 2019

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List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I   **Skoog E**[*], Shin J. H[*], Saez-Jimenez V[*], Mapelli V, Olsson L (2018). Biobased adipic acid – The challenge of developing the production host. *Biotechnology Advances*. DOI: 10.1016/J.BIOTECHADV.2018.10.012

II   **Karlsson E**[‡*], Shin J. H[*], Westman G, Eriksson L. A, Olsson L, Mapelli V (2018). *In silico* and *in vitro* studies of the reduction of unsaturated α,β bonds of *trans*-2-hexenedioic acid and 6-amino-*trans*-2-hexenoic acid – Important steps towards biobased production of adipic acid. *PLOS ONE*, 13(2), e0193503. DOI: 10.1371/journal.pone.0193503

III   **Karlsson E**[‡], Mapelli V, Olsson L (2017). Adipic acid tolerance screening for potential adipic acid production hosts. *Microbial Cell Factories*, 16(1), 20. DOI: 10.1186/s12934-017-0636-6

IV   **Skoog E**, Ferone M, Montriwat P, Mapelli V, Olsson L (2019). ATP levels and membrane lipid composition give insights to the difference in adipic acid tolerance in *Candida viswanathii* and *Saccharomyces cerevisiae. Manuscript*

[*]These authors contributed equally.
[‡]The former name of the author was Karlsson.

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Author's contributions

Paper I: First author (shared). I initiated the study, collected, evaluated and summarized most of the literature on direct pathways to adipic acid, and wrote major parts of the manuscript.

Paper II: First author (shared). I conceptualized and designed the study, and planned and performed the experimental *in vitro* work, including setting up the enzymatic assay. I wrote a major part of the manuscript.

Paper III: First author. I designed the study, performed the experimental work, analyzed the data and wrote the manuscript.

Paper IV: First author. I designed the study and performed the experimental work, with some support with the fermentations. I analyzed the data and wrote the manuscript.

Contents

1. Introduction

Society today relies greatly on non-renewable, fossil-based raw materials for the production of energy, materials and chemicals. The combustion of these raw materials has increased the amount of greenhouse gases in the atmosphere, leading to changes in the climate and the world as we know it today (Figueres et al., 2017). The reduction of greenhouse gases in the atmosphere is therefore of the utmost importance. In EU the vision is that greenhouse gas emissions should be reduced by 80 %, compared with 1990 levels, by 2050, with a target of at least 40 % by 2030 (European Commission, 2018). The target in Sweden is even more ambitious: net zero emission of greenhouse gases to the atmosphere by 2045 (The Government of Sweden, 2015). If these targets are to be attained, alternative, sustainable solutions must be found in all sectors, including transport, chemical production and electricity generation. Replacing non-renewable raw materials with renewable ones is the key. The most abundant renewable raw material on earth is lignocellulosic biomass, found in all trees and plants (Lopes, 2015). Lignocellulosic biomass can be converted into more valuable products in a so-called biorefinery. After pretreatment, sugars are derived, which can then be converted by microorganisms into biofuels, such as ethanol, or building blocks (chemicals) for the production of materials. The work described in this thesis was part of a project called BioBUF, an acronym for Biobased BUlk and Fine chemicals (www.BioBUF.se). The goal of the BioBUF project was to evaluate the potential of a Swedish biorefinery concept utilizing domestic raw materials, including forest residues, for the production of bulk and fine chemicals. The research described in this thesis is introduced below by explaining how the research questions were defined and approached in order to fulfill the main objective.

The aim of this research was to engineer a microorganism for direct production of adipic acid from glucose, more specifically from glucose derived from lignocellulosic raw materials. To realize this, several research questions were identified. Each question is presented below, together with the approaches taken to solve them. An illustration over the areas the research questions in this thesis covers are seen in Figure 1.

METABOLIC PATHWAY   ENZYMES   MICROBIAL HOST
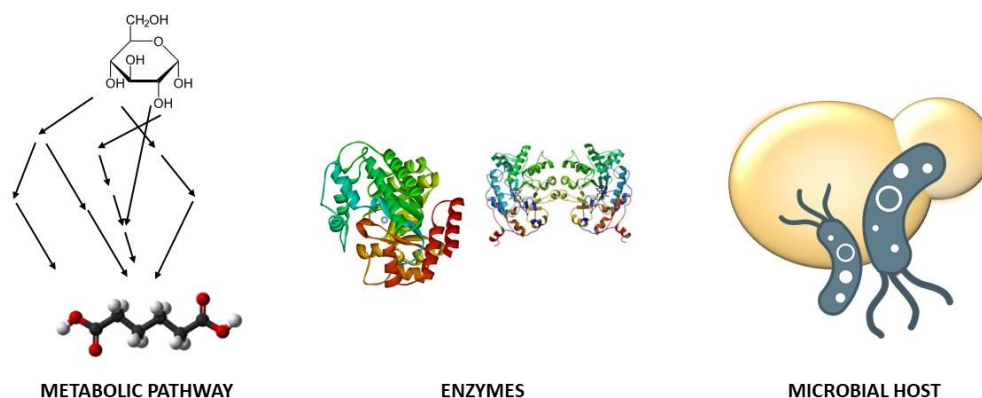
Figure 1. Illustration over the areas covered by the addressed research questions. First, a metabolic pathway, from glucose to adipic acid, was selected. Second, identification of enzymes capable of performing the desired biochemical reactions of the selected pathway. Third, identification of a suitable microbial host for adipic acid production and identification of physiological traits conferring the host with tolerance to adipic acid was addressed.

1. Which metabolic pathway would be suitable for adipic acid production from glucose?

Since there is no known naturally occurring efficient producer of adipic acid, there is no known natural metabolic pathway for adipic acid production. In addition, there are only a few reports on adipic acid production from glucose through genetically modified metabolic pathways, and the resulting titers are very low (Babu et al., 2015; Burgard, Pharkya, and Osterhout, 2012; Deng and Mao, 2015; Kallscheuer et al., 2016; Turk et al., 2015; Yu et al., 2014). To address this research question, a thorough theoretical study was made of all the metabolic pathways described in the literature for the production of adipic acid (**Paper I**). Important aspects, including the maximum theoretical yield, redox balance and the inclusion of biochemical reactions with as yet unidentified enzymes, were evaluated and compared for all the pathways identified. Based on this evaluation, a pathway via lysine was selected as it was redox-balanced and a lysine-overproducing microorganism, *Corynebacterium glutamicum*, is known. The main challenge of this pathway was that it included biochemical reactions with as yet unidentified enzymes in three of the four biochemical reactions. This led to the formulation of the second and third research questions.

2. Can the lysine pathway be further developed in order to obtain a more promising alternative for adipic acid synthesis?

The two databases, KEGG (Kyoto Encyclopedia of Genes and Genomes) and BRENDA (Braunschweig Enzyme Database) were mined, together with the available literature, for

enzymes. In the first search, lysine was the substrate. In the next search, the product of identified enzymes acting on lysine was used as the substrate for the next search, and so on. The identified pathway was then theoretically evaluated regarding redox balance and thermodynamics (**Paper II**).

3. How can enzymes be identified and potentially engineered for the realization of a metabolic pathway, when enzymes with the desired function(s) have not yet been identified?

Enzymes capable of performing the desired type of chemical reaction on substrates similar to those in the metabolic pathway via lysine were selected for further studies (**Paper II**). Both *in silico* and *in vitro* studies were performed on selected enzymes. In the first step, the accessibility of the substrate to the enzymatic pocket and the interaction with catalytic residues were evaluated using *in silico* docking techniques. Enzymes showing promising docking results were then recombinantly produced, purified and tested with regard to their activity on target substrates *in vitro*.

Apart from realizing the metabolic pathway, a substantial part of this work focused on identifying a microorganism suitable for genetic engineering for adipic acid production. In general, titers of 50-100 g L^{-1} are required for acids for the process to be economically feasible (Wang, Lin, Xu, Yang, et al., 2016; Warnecke and Gill, 2005). Therefore, the first research question regarding the potential microbial host addressed tolerance to adipic acid. Tolerance is defined here as the capability of the microorganism to maintain its characteristic behavior when exposed to increasing concentrations of adipic acid.

4. Which microorganism(s) tolerate high concentrations of adipic acid?

To address this research question, candidate microorganisms were screened in the presence of increasing concentrations of adipic acid (**Paper III**). The microorganisms investigated included the well-known bacteria: *Escherichia coli* and *Corynebacterium glutamicum,* yeast: *Saccharomyces cerevisiae* and a filamentous fungus: *Aspergillus niger*, as well as the less-known yeasts *Candida viswanathii* and *Zygosaccharomyces bailii*. Apart from being well-known, *C. glutamicum* was selected as it is an industrial producer of lysine (Becker, Rohles, and Wittmann, 2018), the starting substrate in the selected pathway. *C. viswanathii* has already proven successful for adipic acid production with fatty acids as substrate in industrial settings (Beardslee and Picataggio, 2012; Picataggio and Beardslee, 2013), and is thus likely to tolerate high adipic acid concentrations. The food spoilage yeast *Z. bailii* was included as it is known to be highly resistant to other weak acids, such as sorbic, benzoic, acetic and propionic acid (Stratford et al., 2013).

For a biorefinery to be competitive with a petrochemical refinery, the overall production cost must be minimized. Downstream processing can account for a substantial part of the production cost (Cheng et al., 2012). Therefore, process parameters leading to reduced costs in downstream processing are desirable. One way of reducing this cost in acid production is to run the production process at acidic pH, around the pK_a value of the acid (Tsuge et al., 2016). Therefore, the microorganism should preferably be able to tolerate a high concentration of adipic acid at low pH (<pH 5), (adipic acid has a pK_a of 4.4). However,

reducing the pH increases the fraction of undissociated, membrane-diffusible form of acids, causing acid stress on the cell. Therefore, the fourth research question was refined, addressing the tolerance to undissociated adipic acid.

5. Which microorganism(s) tolerate high concentrations of undissociated adipic acid?
To address this research question, candidate microorganisms were screened in the presence of increasing concentrations of undissociated adipic acid obtained by reducing the pH. The non-conventional yeast *C. viswanathii* was found to be the most tolerant to adipic acid, both in terms of total concentration and undissociated adipic acid. The superior tolerance of *C. viswanathii* resulted in the final research question.

6. Why does *C. viswanathii* have a better tolerance to adipic acid than *Saccharomyces cerevisiae*?
To address this research question, two hypotheses were formulated.

I. The respiratory metabolism of C. viswanathii*, theoretically more efficient in the production of ATP per molecule of glucose than the respiro-fermentative metabolism of S. cerevisiae, can generate the ATP required for export of adipate and maintenance of cytosolic pH, without significant reduction in biomass production.*
To test this hypothesis *C. viswanathii* was cultured in controlled bioreactors with up to saturating concentrations of adipic acid, at pH 5 and pH 4. The ATP content of exponentially growing cells was analyzed and compared to that in *S. cerevisiae* cells cultivated under the same conditions (**Paper IV**).

II. The cell membrane of C. viswanathii *is less permeable to adipic acid than the cell membrane of* S. cerevisiae, *and hence the uptake rate is lower.*
To test this hypothesis, the total lipid composition of exponentially growing cells of *C. viswanathii* and *S. cerevisiae,* in the presence of adipic acid, was analyzed (**Paper IV**).

In this thesis, **Chapter 2** is devoted to the BioBUF project. In **Chapter 3**, metabolic pathways for the production of adipic acid from glucose are presented, together with important aspects that must be considered when selecting a metabolic pathway. The selected metabolic pathway, starting with lysine, is described in detail, together with the approaches taken aimed at establishing the biosynthesis of adipic acid from lysine, and the findings made. In **Chapter 4** important characteristics of a microbial producer of adipic acid and the work pursued to identify such a microbial host are presented. Aspects and findings related to ways in which the tolerance of a microorganism to adipic acid can be improved are also discussed in this chapter.

2. The Biorefinery Concept

The threat posed to the world by climate change has led to the realization that we must replace our current "take–make–waste" economy, based on fossil resources, with an economy based on renewable raw materials, where the aim is to mimic the natural, global carbon cycle (Figure 2). An economy based on renewable raw materials, also known as the bioeconomy, has been defined by the Swedish Research Council for Sustainable Development (Formas), as:

"…an economy based on:

- *A sustainable production of biomass to enable increased use within a number of different sectors of society. The objective is to reduce climate effects and the use of fossil based raw materials.*
- *An increased added value for biomass materials, concomitant with a reduction in energy consumption and recovery of nutrients and energy as additional end products. The objective is to optimize the value and contribution of ecosystem services to the economy."* (Annerberg and Ledin, 2012)
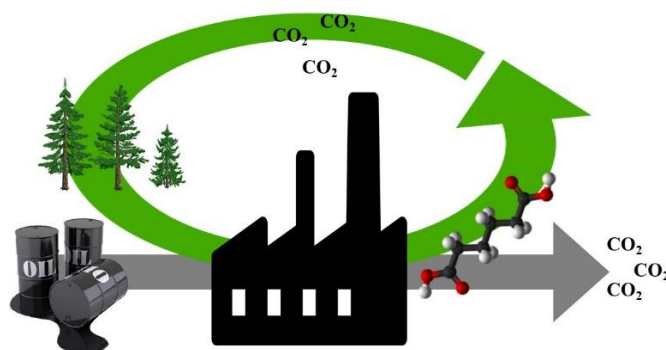

Figure 2. Illustration over the current "take-make-waste" economy based on fossil resources where the carbon dioxide, by the end of a products life cycle, is not recycled (grey arrow) and the bioeconomy based on renewable raw materials, mimicking the natural carbon cycle (green arrow).

Adopting a bioeconomy is important in realizing a sustainable circular economy, the aim of which is to design out waste and pollution, keep products and materials in use and regenerate natural systems (Ellen MacArthur Foundation, 2019). The need for transition to a bioeconomy has been widely recognized, and almost all countries and regions in Europe have included bioeconomy-related aspects in their political agendas (Haarich, 2017). An industrial process important for the development of the bioeconomy is the so-called biorefinery, where renewable raw materials are converted into valuable bulk and added-value products. The general concept of the biorefinery, the feedstocks used, and the products of interest are described below. A more detailed description of the specific biorefinery concept conceived within the BioBUF strategic project, of which this work was a part, is then given

2.1 Introduction to the biorefinery

A biorefinery is similar to a petrochemical refinery, except that the starting materials are of biological origin, stemming from plants or animals. In contrast to a petrochemical refinery, the biorefinery concept also includes sustainability aspects, including environmental, economic and societal factors (Star-COLIBRI, 2011). The definition of a biorefinery given by the International Energy Agency (IEA) is: "the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)" (Van Ree, De Jong, and Kwant, 2011), as illustrated in Figure 3.

Different types of biorefineries exist, in which the biomass may be upgraded via thermal, chemical, enzymatic or microbial conversion, or a combination thereof. In general, there are two distinct types of biorefineries: thermochemical and biochemical (Sandén and Pettersson, 2014). In the thermochemical biorefinery, gasification is the dominant process, whereas fermentation is the dominant process in the biochemical biorefinery. The focus of this thesis is on the biochemical biorefinery, where microorganisms are employed for the conversion of the biomass.
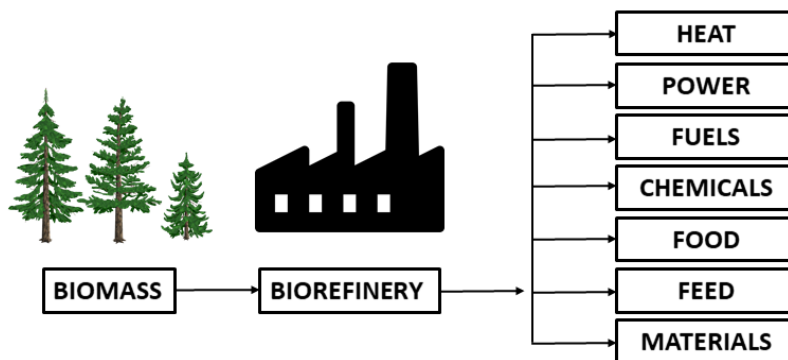
Figure 3. The biorefinery concept: the sustainable processing of biomass into a variety of marketable products and energy.

2.2 Raw materials

The most important biomass feedstocks in a biorefinery are sugars (e.g. from sugar beet or sugarcane), starch (e.g. from wheat and corn), and lignocellulosic biomass (e.g. from forest or agricultural waste, and switchgrass) (Sandén and Pettersson, 2014). In addition to these, oil-based feedstocks are also used (e.g. coconut, palm and palm kernel oil) and organic residues (e.g. industrial and post-consumer waste). Algal biomass has also recently attracted interest as a feedstock in biorefineries (Van Ree et al., 2011).

Lignocellulosic biomass
Lignocellulose, found in plants and trees, is the most abundant type of biomass on Earth (Lopes, 2015). In Sweden, and other countries rich in forest resources, lignocellulosic woody biomass would be the most important feedstock for biorefineries (Sandén and Pettersson, 2014), and would play a key role in developing the Swedish bioeconomy (Person, 2016). Apart from being abundant, lignocellulosic biomass does not compete directly with food and feed production, as is the case when using sugar and starch from food crops. Lignocellulose consists of the polymers cellulose (40-50 %), hemicellulose (20-40 %) and lignin (20-30 %) (Sjöström, 1993), arranged in a complex matrix. The exact distribution of these polymers in woody biomass varies depending on the type of tree and location within the tree (Pauly and Keegstra, 2008). Cellulose consists of long, linear chains of linked glucose units, whereas hemicellulose consists of highly branched chains of both hexoses, i.e. mannose, galactose and glucose, and pentoses, mainly xylose and arabinose. Lignin consists of a highly complex, three-dimensional structure of aromatic species. To obtain fermentable monosaccharides from cellulose and hemicellulose, pretreatment is required to open up the complex structure of the lignocellulosic biomass, followed by enzymatic degradation of the polymers into monosaccharides. Microbial inhibitors can be released from the lignocellulosic biomass depending on the harshness of the pretreatment. Microbial inhibition must therefore be considered when using lignocellulosic raw materials. The lignin part of the lignocellulosic biomass contains aromatic species, which cannot be fermented by microorganisms. Traditionally, the lignin part has been burned to produce heat and energy, but there is now considerable interest in upgrading this lignin fraction to provide more valuable products (Holladay et al., 2007; Ponnusamy et al., 2019).

2.3 Products

The products of a biorefinery can be arranged in a value pyramid, where high-value products are produced at low amounts, and low-value products are produced at high amounts (Figure 4). The benefits of a biorefinery can be increased by integration, in which low-value, high-volume bulk product(s) are co-produced with high-value, low-volume fine product(s). Related to products of a biorefinery, the term "cascading" is often used. No general definition of cascading exists, but it refers to an efficient utilization of the resources where a common theme is that "material use of wood should be prioritized over energy use of wood" (Olsson et al., 2016).
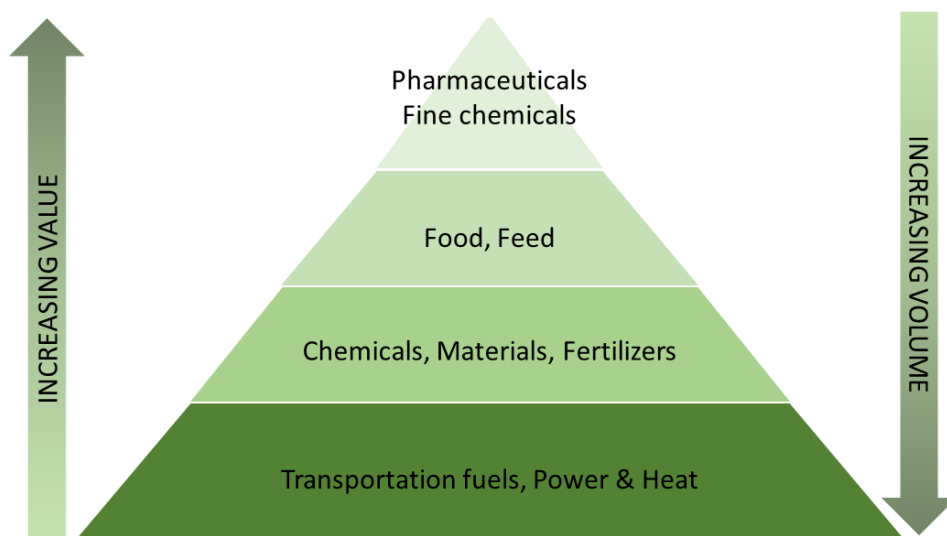
Figure 4. Bio-product value pyramid. The value of the products increases with each step up in the pyramid, whereas the volume decreases (Adopted from Haarich, 2017).

Platform chemicals

Platform chemicals, often called building blocks, are relatively low-molecular-weight molecules used to produce structurally different molecules or polymers via chemical reactions. The functional groups of platform chemicals are responsible for their chemical reactivity. A functional group is a specific moiety within the molecule, e.g. an alcohol, a ketone or a carboxylic acid, capable of forming covalent bonds with other functional groups. The most common kinds of building blocks in the petrochemical industry are olefins (e.g. ethylene and propylene) and aromatics (e.g. benzene, toluene and xylene isomers). A wide range of molecules can be produced from these building blocks, such as solvents and detergents, and polymers used e.g. in the plastics industry. It is necessary to identify potential platform chemicals that can be used to replace petrochemical building blocks in the establishment of biorefineries (Werpy and Petersen, 2004). Biobased platform chemicals can be identical in their chemical structure to their petrochemical equivalents. These platform chemicals have the benefit of already being part of the existing industrial infrastructure, and can thus easily replace their petrochemical equivalents as so-called "drop-in" chemicals. Examples of drop-in chemicals are ethylene and acetic acid, both of which can be obtained from microbially produced ethanol. Some potential platform chemicals have no structurally identical petrochemical counterparts, and further conversion is often required before reaching the market. Examples of such chemicals are succinic acid and polyhydroxyalkanoate. As an example of a platform chemical, succinic acid and some of its downstream products are illustrated in Figure 5.
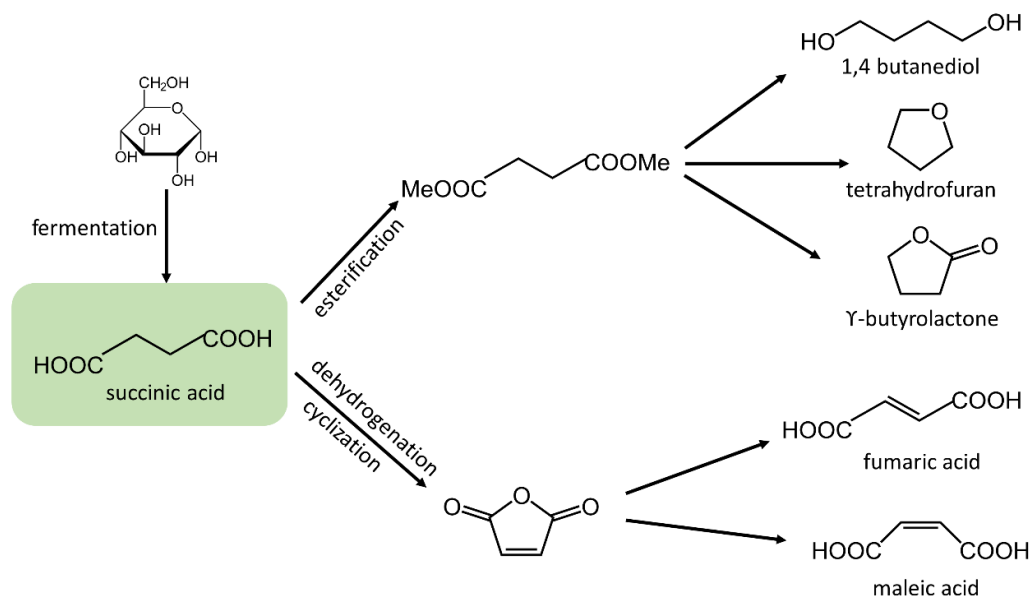
Figure 5. Succinic acid as a platform chemical (adapted from Sandén and Pettersson, 2014).

2.4 The BioBUF concept

The upgrading of renewable domestic raw materials to value-added bulk and fine chemicals for a biobased economy using technology development, systems integration and environmental impact assessment, was evaluated in the BioBUF project. A schematic view of the process, which is further explained below, is given in Figure 6.

Raw materials evaluated in the BioBUF project

The raw materials evaluated were Swedish forest residues and algae biomass. Forest residues (branches, tips and roots) are normally left in the forest after harvest or, at best, used for energy production (Ortiz et al., 2014). The exploitation of forest residues for conversion into value-added products is thus an example of waste stream valorization. The composition of forest residues is highly variable, and depends, for example, on tree species, geographical location, time of year and harvesting method (Kozinski, Dalai, and Nanda, 2013). Furthermore, the cellulose content in branches, including bark, is lower (29-33 %) (Backlund, 2014) than in biomass from the stem (40-50 %) (Sjöström, 1993). Thus, the amount of glucose that can be obtained from residues is lower than from the stem. The high variability in the composition of woody biomass poses a considerable challenge in a biorefinery since the process efficiency is dependent on the raw material. Algae biomass has also been included as an alternative in the BioBUF project. The use of algal biomass as a source of fermentable sugars to produce adipic acid was found to be unfeasible based on modeled areal yields versus fermentation requirements. However, it has been suggested that algal biomass could be used as a source of nutrients and for the production of high-value products such as lutein (Mayers et al., 2016).
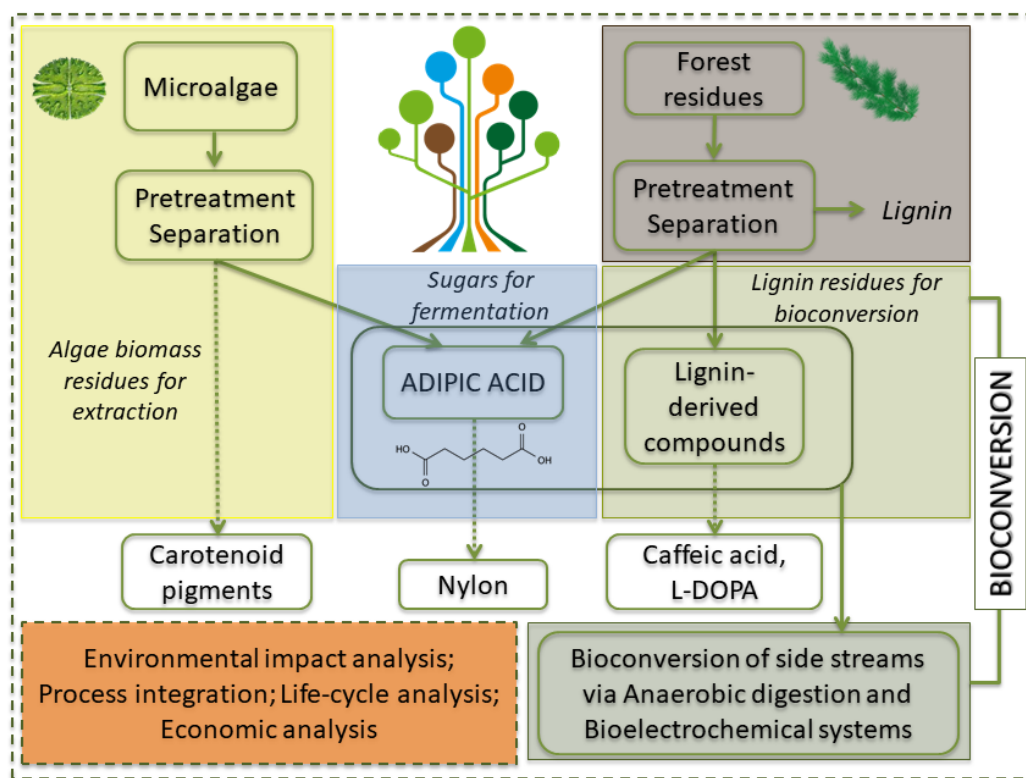
Figure 6. Schematic of the process scheme for the BioBUF biorefinery concept.

Products considered in the BioBUF project

The bulk product considered in the BioBUF concept was adipic acid, produced via microbial conversion of the fermentable sugars from the biomass. Adipic acid has been identified as a potential biobased platform chemical owing to its use in several industries for the production of a variety of products (Van Ree et al., 2011; Werpy and Petersen, 2004) (Figure 7). Adipic acid consists of six carbon atoms, arranged in a straight chain, with the functional group, carboxylic acid, at both ends. The current global annual production is close to 3 million tonnes, with a market value of 6 billion USD and a compound annual growth rate of 3-5 % (Taylor et al., 2015). Most of the adipic acid produced is used in the nylon industry, where it is used together with hexamethylenediamine to make nylon 6.6. The technological development required for the microbial production of adipic acid is the subject of this thesis and is discussed in detail in the following chapters.

In addition to the production of the bulk chemical adipic acid, integrated processes for the production of high-value products from lignin and algal biomass were also evaluated. Soluble phenolic compounds originating from the lignin stream were surveyed for their potential to produce targeted aromatic products of high market value, such as caffeic acid and L-DOPA through the development of biochemical conversion techniques. The extraction of lutein from algae biomass was evaluated and compared with the current production method from the marigold flower. No biochemical conversion techniques for the targeted products could be established for the lignin stream within the time frame of the

project. However, upgrading of both the lignin fraction and algal biomass is believed to be a key factor in establishing a competitive biorefinery.
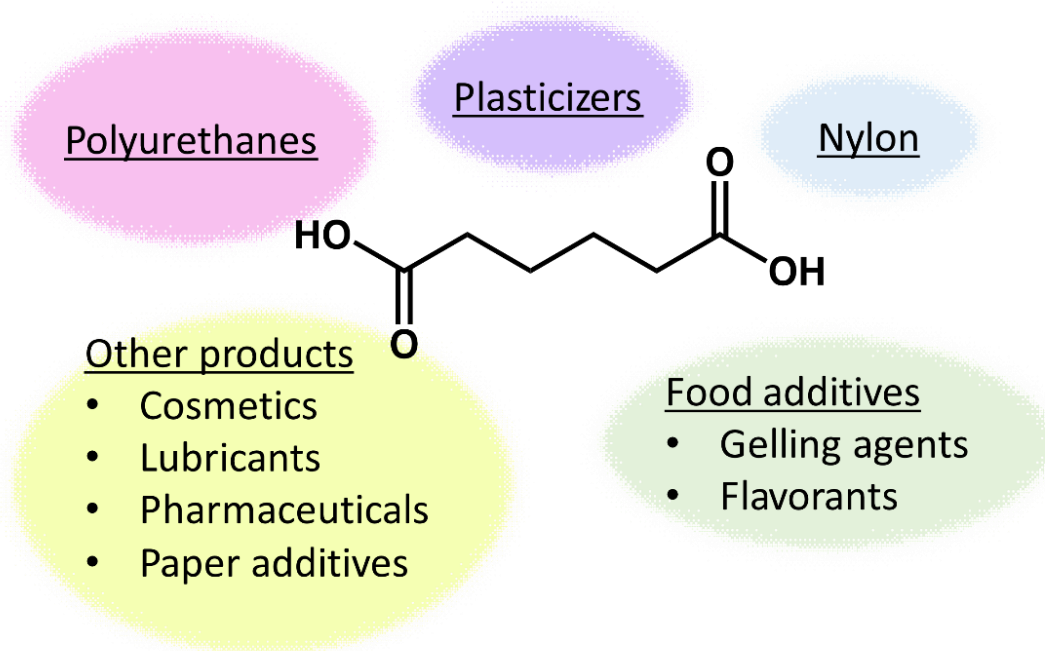
Figure 7. Structure of adipic acid and examples of its uses.

Bioconversion of side streams via anaerobic digestion and bioelectrochemical systems
In anaerobic digestion, biodegradable material is converted by microorganisms into biogas in the absence of oxygen. In the BioBUF project, the anaerobic digestion of cow manure and was evaluated, both as a source of nutrients and for heat and power production by combustion of the biogas produced. The nutrients could be used in both the fermentation process and in microalgae biomass production where it can have a significant impact on cost and environmental burden. For instance, effluent from anaerobic digestion of food waste could cover 100 % of the nitrogen demand and up to 32 % of the phosphorous requirement for cultivation of the marine microalga *Nannochloropsis sp*. Thereby the cost for and environmental impact of nitrogen and phosphorous could be reduced by up to 90 % versus the use of artificial fertilizers (Mayers et al., 2017). The electricity and heat produced from the biogas could be used elsewhere within the biorefinery, thereby reducing the need for external energy input, potentially coming from petrochemical sources.

An additional way to make use of by-products of the BioBUF biorefinery was suggested to be the use of bioelectrochemical systems, in which organic or inorganic substances could be oxidized or reduced with the aid of microorganisms capable of transferring electrons between the substance and an electrode. The potential for improved lysine production from glucose using an engineered lysine-producing strain of *Corynebacterium glutamicum* in a bioelectrochemical setup, has been investigated (Xafenias, Kmezik, and Mapelli, 2017). The study demonstrated that under reducing conditions, in the presence of CO_2 and of a redox mediator, the yields and titers of lysine could reach levels that have only been achieved under

aerobic conditions. Ideally, the lysine produced could be fed into the fermentation step for subsequent conversion to adipic acid.

Systems analysis and life-cycle assessment

Evaluation of the overall system led to the identification of outputs from the microbial conversion to adipic acid (water, nutrients, CO_2 and residual heat) that could be recycled to other processes. However, some streams contain impurities, which could limit valorization. Replacing petrochemical-derived adipic acid with biobased adipic acid could significantly reduce the environmental impact. However, the performance of the conceptual biorefinery is heavily dependent on the background energy system and heat production, regardless of the configuration used. Here, the use of biomass for heat production, can reduce the global warming potential with up to 70 % compared with heat being provided by the average Swedish energy mix. The use of forest residues for adipic acid production only contributes with 1-4 % in global warming potential. The use of biomass for heat production is thus crucial to ensure a low environmental impact. In addition, it was found that the profitability of the biorefinery is likely to be dependent on revenues from by-products, such as lutein.

Conclusions from the BioBUF project

Considerable knowledge has been obtained on a highly integrated biorefinery concept, where Swedish forest residues and algae biomass could be used as feedstocks for the co-production of bulk and fine chemicals. Although the optimal configurations of products and processes have probably not yet been identified for a biorefinery based on Swedish raw materials, the results highlight the importance of co-producing both low- and high-value products. In agreement with other studies, valorization of the entire lignocellulosic feed-stock, including the lignin fraction, together with integrated processes, e.g. energy production for use within the biorefinery, and co-production of high-value products, are key to making the biorefinery competitive with its petrochemical counterpart (Johnson and Hart, 2016; Rauschen et al., 2015). Considerable efforts are still required in terms of technical development and the logistics of materials, infrastructure and location of such a biorefinery. Nevertheless, the realization of biorefineries such as the one conceptualized in the BioBUF project is essential for the transition to a bioeconomy.

3. Principles of Metabolic Engineering Applied to Adipic Acid Biosynthesis

3.1 Metabolic pathway selection – Aspects to consider

All biochemical reactions that takes place in the living cell are performed by enzymes. Each chain of enzymatic reactions, where the product from one enzymatic reaction constitutes the substrate for the next, is known as a metabolic pathway. The term "metabolic engineering" emerged in the 1990s and has been defined as: "The directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology" (Stephanopoulos, Aristidou, and Nielsen, 1998). Initially, the aim was to maximize the production of naturally produced compounds of interest. The main approaches used were to "copy and paste" genes from one species to another, and the deletion of genes encoding competing pathways. Since then, the metabolic engineering toolbox has developed dramatically, and numerous approaches are now available, allowing the production of non-endogenous products, through the generation of novel pathways (Lee et al., 2012; Yim et al., 2011). Today, metabolic engineering techniques have been successfully applied to engineer microbial cells for improved product formation of an array of products, including bulk chemicals (Okino et al., 2008), biofuels (Buijs, Siewers, and Nielsen, 2013), pharmaceuticals (Baeshen et al., 2014) and food additives (Rahman, 2015).

A substantial part of the work presented in this thesis was dedicated to the identification of a metabolic pathway for the synthesis of the non-natural product adipic acid. In this section, the aspects that should be considered when selecting a metabolic pathway in order to achieve the high titer, productivity and yield necessary for an industrially viable process will be outlined. Box 1 summarizes these aspects.

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Box 1. Aspects that should be considered when selecting a metabolic pathway

- Maximum theoretical yield
- Redox reactions
- Availability and catalytic efficiency of enzymes
- Thermodynamics
- Pathway length
- Availability of starting metabolites

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Maximum theoretical yield

The maximum theoretical yield, Y_{max}, is a theoretical measure of the quantity of product that can be obtained by complete conversion from the limiting reactant in a chemical reaction. Following this definition, the Y_{max} for adipic acid from glucose would be 100 %. However, the Y_{max} calculated from biochemical reactions in a cell is reduced compared to the Y_{max} of a chemical reaction. This is due to that product formation in the cell is linked with formation of unavoidable by-products for the necessary generation of e.g. ATP and redox balance. Here, genome-scale models are important tools when evaluating the potential of a certain metabolic pathway in a specific host organism. In these models, the metabolism is reconstructed from all the known metabolic reactions in the specific organism (O'Brien, Monk, and Palsson, 2015). The first genome-scale metabolic model was published at the end of the last millennium (Edwards and Palsson, 1999), and an increasing number of models have since been developed for the evaluation of metabolic pathways and their potential in the host of interest (Oberhardt et al., 2011; Österlund, Nookaew, and Nielsen, 2012). Several different models may exist for a particular organism, each based on the assumptions and mathematical calculations applied by the developer. For instance, at least 25 models have been published for *Saccharomyces cerevisiae* (Heavner and Price, 2015). Therefore, when comparing the maximum theoretical yields of different pathways, it is important to bear in mind that they are theoretical, and are determined by the underlying assumptions and calculations on which the model is based.

Redox reactions

Enzymatic reactions often involve the transfer of electrons from one compound to another. The compound gaining electrons is reduced, whereas the compound losing electrons is oxidized, and the overall reaction is termed a redox reaction. Several kinds of electron carriers are present in cells. Among those mainly responsible for electron transfer in metabolic redox reactions are NADH and NADPH. During redox reactions, these cofactors are either in their reduced form: NADH/NADPH, carrying an electron and acting as electron donors, or in their oxidized form: $NAD^+/NADP^+$, ready to accept an electron. Balancing these reactions is important to maintain a high flux through the pathway and avoid the loss of carbon via other pathways used to balance the overall cellular redox potential. For example, a strain of *S. cerevisiae* engineered so that it can ferment xylose exhibits reduced product yield due to an imbalance in the reducing power. This xylose-fermenting strain expresses the genes *XYL1* and *XYL2* from *Pichia stipitis* encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, which catalyze the first two steps in the

xylitol pathway (Kötter et al., 1990; Kötter and Ciriacy, 1993). XR can use both NADPH and NADH, but has a higher affinity for NADPH; whereas XDH uses only NAD^+ (Kötter et al., 1990; Rizzi et al., 1988). This difference in cofactor usage results in an imbalance when $NADP^+$, formed by the reduction of xylose by XR, cannot be used by XDH for the oxidation of xylitol. Xylitol is thus excreted into the fermentation broth, causing a loss of carbon and a reduction in the product yield (Kötter et al., 1990; Kötter and Ciriacy, 1993). Enzyme engineering of XR to change its specificity for NADPH to NADH has led to improvements in the ethanol yield (Petschacher and Nidetzky, 2008), demonstrating the importance of balancing the redox potential of a pathway.

Availability and catalytic efficiency of enzymes
The availability and catalytic efficiency of the enzymes involved in a metabolic pathway are key to successful product formation. For an enzyme to be available, the enzyme with desired specificity must be expressed in its active form when required. Furthermore, it has to be produced at the optimal amount and localized in the right cell compartment, i.e. where the substrate of the enzyme is found. The factors determining the catalytic efficiency of an enzyme are the turnover number and the Michaelis constant. The turnover number, k_{cat}, is the catalytic constant for the conversion of the substrate into the product, and is expressed in s^{-1}. The Michaelis constant, K_M, is defined as the substrate concentration at which the reaction rate is 50 % of its maximum rate, and describes the affinity of the enzyme for the substrate. A high value of k_{cat} and a low value of K_M are thus desirable. Each enzymatic reaction in the metabolic network is a balance between the forward and backward fluxes, which are determined by the kinetics of the enzyme, the amount of the enzyme, and the concentrations of the substrate and product (Noor, 2012). As the kinetics of different enzymes differ in a particular pathway, an efficient enzyme can drive the reaction of a less efficient enzyme by decreasing the product concentration from the chemical equilibrium towards product formation for the latter.

Product inhibition, i.e., inhibition of the enzyme by the reaction product(s), is another important factor that should be taken into consideration when evaluating enzymes. Overcoming product inhibition can increase the reaction rate and final titer (Kim et al., 2019; Schügerl and Hubbuch, 2005).

Enzymes with the desired properties, i.e., those capable of performing the desired reaction with a high catalytic efficiency, can be identified, for example, in the enzyme database BRENDA, which currently includes ~84 000 manually curated entries (Jeske et al., 2019). The number of enzymes is increased dramatically, to over 60 million, when the number of gene sequences in the UniProt database (www.uniprot.org) is included (Gerlt, 2016). These databases offer a golden opportunity for researchers to mine for enzymes with specific properties. However, the functions of ~50 % of the gene sequences in UniProt may be incorrect, uncertain or unknown, due to *in vitro* activities of uncharacterized enzymes being assigned based on sequence homology alone (Gerlt, 2016). Thus, the potential to mine the UniProt database for a specific enzyme is limited. Despite the huge number of known and putative enzymes, novel metabolic pathways including biochemical reactions with yet

unidentified enzymes are still being suggested. In fact, one of the major hurdles in the application of industrial biocatalysis to chemical synthesis is the lack of the enzymes required to catalyze the reactions leading to a viable process (Kaul and Asano, 2012). One example of a novel pathway containing as yet unidentified enzymes is the pathway to adipic acid via lysine, which is further described in Section 3.2. The identification of enzymes capable of performing catalytic reactions yet not demonstrated by an enzyme, requires a great deal of work, and can include several rounds of screening combined with enzyme engineering of enzyme candidates.

Thermodynamics
In accordance with the second law of thermodynamics, the change in Gibbs free energy must be negative for enzyme reactions or overall pathways to proceed (Jones and Atkins, 2003). Although enzymes can lower the activation energy, they cannot change the Gibbs free energy of a reaction. Evaluating the thermodynamics of a specific reaction, metabolic pathway or network, can determine the reaction directionality and provide an estimate of how far from, or close to, equilibrium the reactions in the network operate (Ataman and Hatzimanikatis, 2015). The different approaches used to analyze the thermodynamics of metabolic pathways have been reviewed by Ataman and Hatzimanikatis (2015). The thermodynamic parameters of biological systems are often obtained using the group contribution method, where thermodynamic parameters are estimated based on chemical structures, rather than experimental data (Noor et al., 2013). Knowledge on metabolite concentration, pH and ionic strength are required when calculating the thermodynamics (Noor et al., 2014; Park et al., 2016). Such parameters are often not readily available and must be estimated or calculated from known variables, e.g., the glucose concentration in the medium, and the kinetics of the enzymes and products obtained. Despite the fact that the thermodynamic constraints of a pathway are often obtained from calculations and estimates, they can provide valuable information and guidance in assessing the feasibility of a certain pathway or identifying bottlenecks that must be overcome.

Pathway length
Each bioconversion step in the pathway requires resources for the synthesis of amino acids and the assembly of enzymes. Therefore, if several pathways exist, it may be beneficial to choose the one with the least number of bioconversion steps (Henry, Broadbelt, and Hatzimanikatis, 2010).

Availability of starting metabolite(s)
The flux through a pathway should be high, avoiding or minimizing the channeling of carbon to other pathways and by-products. The flux then depends on the rate of formation of the starting metabolite(s). The selection of a pathway is therefore interlinked with the metabolism of the target microorganism to be engineered. Thus, a pathway in which the flux of the starting metabolite(s) is high for the intended microorganism should preferably be chosen. For instance, if a pathway starts with lysine, a microorganism known to overproduce lysine should be chosen. The start of some pathways includes the condensation of two molecules, requiring equimolar concentrations of these two metabolites. This is the case for

some of the direct pathways to adipic acid, as discussed in Section 3.2. The flux through the pathway is thus determined by the metabolite(s) with the slowest formation rate. It is thus necessary to identify bottlenecks in the pathway and increase the formation rate of the metabolite responsible for the reduced flux.

3.2 Metabolic pathways for producing adipic acid from glucose

To the best of the author's knowledge there is no known natural producer of adipic acid. However, adipic acid production has been reported in the cell lysate of the bacterium *Thermobifida fusca* with the addition of the endogenous enzyme 5-carboxy-2-pentenoyl-CoA reductase encoded by *Tfu_1647* as well as addition of the starting metabolites of the pathway: acetyl-CoA and succinyl-CoA (Deng and Mao, 2015). This indicates that *T. fusca* has a natural capacity for adipic acid production, although the endogenous pathways for acetyl-CoA and succinyl-CoA, enzyme efficiency and/or expression levels of 5-carboxy-2-pentenoyl-CoA reductase must be improved. Due to the lack of natural metabolic pathways for the production of adipic acid, efforts have been made to design novel pathways for the production of this acid (Burgard et al., 2012) and some proof-of-concept results, showing adipic acid production from glucose metabolism, have been reported (Babu et al., 2015; Kallscheuer et al., 2016; Turk et al., 2015; Yu et al., 2014). In the present work, all the publicly available metabolic pathways for the production of adipic acid, both direct and indirect, were identified and evaluated with respect to their advantages and disadvantages, in accordance with the important aspects described in Section 3.1 (**Paper I**). Three models for calculating the Y_{max} reported in Table 1 were found in the literature. The one reported by Burgard et al. (2012) is based on a stoichiometric network of *E. coli* similar to the model *i*JR904 (Reed et al., 2003). The models of *E. coli* and *S. cerevisiae* reported by Averesch et al. (2018) were built on previously reported models of the same author (Averesch and Krömer, 2014).

It is worth mentioning that the highest titers of bio-based adipic acid reported so far have been obtained using fatty acids, such as palm oil, as raw material, using an engineered strain of the yeast *Candida* as the microbial host (Beardslee and Picataggio, 2012), or glycerol with an engineered strain of *E. coli* (Zhao et al., 2018), reaching 50 g L^{-1} and 68 g L^{-1}, respectively. Although these are very interesting results, the focus of the present work is on adipic acid production from glucose, and adipic acid production using other substrates will therefore not be further discussed. However, these reports raise the question of whether it is a good strategy to produce adipic acid from glucose, or whether it is better to use more reduced substrates.

Indirect pathways for adipic acid production
Although the aim of the BioBUF project was the direct production of adipic acid, a small section of this thesis is devoted to the indirect pathways of adipic acid production. (The indirect metabolic pathways for the production of adipic acid can be found in Figure 6 and Figure 7 in **Paper I**.) The products of the indirect pathways, *D*-glucaric acid and *cis,cis*-muconic acid, can be efficiently converted to adipic acid via chemo-catalytic hydrogenation

Table 1. Comparison of direct and indirect metabolic pathways for the production of adipic acid from glucose. The comparison includes both the theoretical maximum yield, Y_{max}, as given by two different stoichiometric network models of *E. coli* and one of *S. cerevisiae*, and values reported ($Y_{reported}$) for the best strain producing adipic acid, *cis,cis*-muconic acid or glucaric acid from glucose, depending on the pathway.

Pathway	$Y_{max}^{1,a}$ (mol/mol)	$Y_{max}^{1,b}$ (mol/mol)	$Y_{max}^{1,c}$ (mol/mol)	$Y_{reported}^{1}$ (mol/mol)	Titer[1] (g/L)	$r_{adipic\ acid}^{1}$ (g L^{-1} h^{-1})	Host species	Limitations	Ref.
Direct production of adipic acid									
Reverse adipate degradation	0.92	0.92	0.92/0.90	0.054	2.23	0.034	*T. fusca*	Redox imbalance Need for equimolar concentrations of starting metabolites	[1]
Reverse β- followed by ω-oxidation	n.r.	n.r.	n.r.	n.r	n.r	n.r	n.r	Redox imbalance Need for equimolar concentrations of starting metabolites	[2]
3-oxoadipic acid	0.92	0.92	0.92/0.90	n.r.	n.r.	n.r.	n.r.	Need for equimolar concentrations of starting metabolites Unidentified enzymes	[3,4]
2-oxopimelic acid	n.r.	0.90/0.85	0/0.32	n.r.	0.3	<0.01	*E. coli*	Redox imbalance Unidentified enzymes	[5]
2-oxoadipic acid	0.67 (0.45)	0.90/0.85	0/0.38	n.r.	n.r.	n.r.	n.r.	Redox imbalance Unidentified enzymes	[3,4]
Lysine via 2-oxoglutaric acid "the yeast pathway"	0.40 (0.20)	n.a.	0.67/0.51	n.r.	n.r.	n.r.	n.r.	Unidentified enzymes	[3,4]
Lysine via aspartic acid "the bacterial pathway"	0.50 (0.34)	0.92/0.91	n.a.	n.r.	n.r.	n.r.	n.r.	Unidentified enzymes	[3,4]
Indirect production of adipic acid									
Muconic acid									
3-dehydroshikimate	0.75	0.88/0.84	0.85	0.22	36.8	0.76	*E. coli*	Oxygen-requiring enzymes	[6]
Glucaric acid									
D-glucuronic acid	n.r.	~1	~1	0.13	1.13	0.016	*E coli*	Oxygen-requiring enzymes	[7]

[1] Yields are expressed as mol product/mol glucose. Titers are given as final concentration of product, r_p is the volumetric production rate of product.
[a] Yields are taken from Burgard et al., (2012) and are based on a stochiometric network of *E. coli*. If anaerobic condition affects Y_{max}, these are reported in parentheses.
[b] Yields are taken from Averesch et al (2018) and are based on a model of *E. coli*. If two values are given, these are reported as w/o biomass/w biomass.
[c] Yields are taken from Averesch et al (2018) and are based on a model of *S. cerevisiae*. If two values are given, these are reported as w/o biomass/w biomass.

n.r. = not reported, n.a. = not applicable

[1] Deng and Mao, 2015, [2] Clomburg et al., 2015, [3] Burgard et al., 2012, [4] Averesch et al., 2018, [5] Turk et al., 2015, [6] Niu et al., 2002, [7] Moon et al., 2009

(Niu, Draths, and Frost, 2002; Vardon et al., 2015), and these pathways are therefore of interest for biobased adipic acid production. During recent decades, considerable effort has been devoted to the development of pathways to glucaric and muconic acid (Chen et al., 2018; Curran et al., 2013; Draths, Frost, and Indiana, 1994; Johnson et al., 2016; Leavitt et al., 2017; Moon et al., 2009; Niu et al., 2002; Vardon et al., 2015). Interestingly, it has been reported that lignin-derived aromatic species can be converted to muconic acid using an engineered *Pseudomonas putida* strain. A molar yield of 67 %, based on the consumption of *p*-coumarate and ferulate, was reported after 24 hours of cultivation in a shake flask containing lignin derived from corn stover (Vardon et al., 2015). This result opens up the possibility of utilizing the lignin fraction for indirect adipic acid production.

Assuming an almost 100 % conversion efficiency to adipic acid, the highest yields reported so far for glucaric and muconic acids would result in the highest yields of adipic acid from glucose reported to date (2019-05-09) (Table 1) (Moon et al., 2009; Niu et al., 2002). Unfortunately, the additional chemical step, requiring an expensive catalyst and harsh process conditions with high pressure, increases the overall production cost (Warnecke and Gill, 2005), thereby limiting the industrial feasibility of these pathways.

Direct pathways for adipic acid production
Six main direct pathways for the production of adipic acid from glucose, with some variants, were identified in the literature (Figure 8). The details of each pathway, including the metabolites and enzymatic reactions, are described in detail in **Paper I**. In this part of the thesis, the focus will not be on specific details, but rather on a comparison of the pathways regarding their potential for adipic acid production, together with some main findings from **Paper I**.

Among the six identified adipic acid pathways, only two have been reported to produce adipic acid from glucose: the reverse adipate degradation pathway (Figure 8, blue) and the pathway via 2-oxopimelic acid (Figure 8, purple). The reverse adipate degradation pathway was the first pathway to be proven for adipic acid production, using an engineered strain of *E. coli* (Yu et al., 2014). Since then, this pathway has attracted considerable attention resulting in the highest titer of 2.23 g L^{-1} reported to date (2019-05-09), using an engineered strain of the bacterium *T. fusca* (Deng and Mao, 2015). However, the yield achieved was low, reaching only 0.054 mol adipic acid per mol of glucose (Table 1). For the 2-oxopimelic pathway an engineered strain of *E. coli* was reported to produce adipic acid with a titer of 0.3 g L^{-1} and a productivity of less than 0.01 g L^{-1} h^{-1}. The obtained yield was presumably very low as it was not reported (Table 1) (Turk et al., 2015).

Different values for the theoretical Y_{max} of the pathways starting from 2-oxoglutaric acid: 2-oxopimelic acid and 2-oxoadipic acid pathways (Figure 8, purple and yellow) are reported for the different models used. For the model based on *S. cerevisiae*, adipic acid production was stoichiometrically impossible without biomass formation (Averesch et al., 2018). This was not observed in the *E. coli* models. The reason for this is that during formation of the precursor 2-oxoglutaric acid, NADPH is produced. Since *S. cerevisiae* does not harbor

transhydrogenases like *E. coli*, the major sink for cytosolic NADPH is biomass, resulting in lower Y_{max} for *S. cerevisiae* than in *E. coli*.

Adipic acid production via lysine can occur via two main metabolic routes, starting with either 2-oxoglutaric acid (also known as α-ketoglutarate) or aspartic acid (Figure 8). The first pathway, starting with 2-oxoglutaric acid, is native to yeasts such as *S. cerevisiae*, where the pathway partially takes place in the mitochondria (Averesch et al., 2018), but it is not found in *E. coli*. Most bacteria instead use the pathway starting with aspartic acid, for lysine synthesis (Figure 8). For simplicity, the lysine pathway starting with aspartic acid is referred to below as the "bacterial pathway" and the lysine pathway starting with 2-oxoglutaric acid as the "yeast pathway". The value of Y_{max} for adipic acid production via lysine depends on the pathway used, and on the presence or absence of oxygen. Higher Y_{max} are reported for the bacterial pathway (0.91 mol adipic acid per mol glucose) than for the yeast pathway (0.67 mol adipic acid per mol glucose). (Table 1) (Averesch et al., 2018). There are two reasons for the lower yield calculated for the yeast pathway. The first is that the partial mitochondrial localization of the pathway in yeast requires additional energy for the transport of metabolites, and the second is that the formation of the precursor 2-oxoglutarate from the TCA cycle involves the production of CO_2 i.e. a loss of carbon (Averesch et al., 2018). In addition, anaerobic conditions reduce the value of Y_{max} (Burgard et al. 2012), and aerobic conditions are thus preferable. It should be noted that the reported values for Y_{max} via the lysine pathways differ between the models used, even between the two models of *E. coli* (Table 1). This shows that the different assumptions and calculations of the models can have a huge impact on the calculated Y_{max}.

Values of Y_{max} above 0.90 have been reported for the reverse adipate degradation and the 3-oxoadipic acid pathways, irrespective of the model used. It should be mentioned that thermodynamic calculations of the pathways for both *E. coli* and *S. cerevisiae* using physiologically relevant pH values, showed that these pathways have at least one infeasible reaction. However, only the 3-oxoadipic acid pathway in *S. cerevisiae* was found to be completely infeasible due to contradicting restrictions in metabolite concentrations required for the succinyl-CoA:acetyl-CoA transferase and the subsequent 3-oxoadipyl-CoA transferase, preventing the functionality of the pathway (Averesch et al., 2018).

Four of the pathways and their variants suffer from redox imbalance (reverse adipate degradation, reverse β- followed by ω-oxidation, 2-oxopimelic acid, 2-oxoadipic acid). The low yields obtained for the reverse adipate degradation pathway and 2-oxopimelic acid pathways could be a direct consequence of the redox imbalance. Given that glycerol has a higher degree of reduction than glucose, the generation of reducing equivalents is doubled per carbon mol during the formation of acetyl-CoA from glycerol than from glucose (Clomburg et al., 2015). Therefore, the higher yield reported when using glycerol as the substrate (0.24 mol adipic acid per mol glycerol) (Zhao et al., 2018) than the highest yield on glucose (0.054 mol adipic acid per mol glucose) (Deng and Mao, 2015) is not surprising. To achieve higher yields on glucose, the generation of reducing equivalents coupled to the formation of adipic acid is required to avoid carbon loss.

Figure 8. Overview of the metabolic pathways for the direct production of adipic acid. The pathway to lysine is either via aspartic acid, i.e. in bacteria, or via 2-oxoglutaric acid i.e. in yeast. Enzymatic activity has been demonstrated for the enzymatic steps indicated by full lines, while no proof of enzymatic activity has been demonstrated for reactions indicated by dashed lines

In three of the cases (reverse adipate degradation, reverse β- followed by ω-oxidation and 3-oxoadipic acid) the pathway starts with the condensation of two metabolites (Figure 8). Therefore, equimolar concentrations of the starting metabolites are required to ensure a steady flux through these pathways.

The greatest challenge in five of the reported pathways and their variants (2-oxopimelic acid, 3-oxoadipic acid, 2-oxoadipic acid and the lysine pathways) is the lack of identified enzymes for one or more of the reactions in the pathway. In fact, adipic acid production has only been reported for the 2-oxopimelic acid pathway. In this case, adipic acid was produced in *E. coli* as an unintended by-product due to the adipate semialdehyde being oxidized into adipic acid instead of being converted into the intended product 6-aminocaproic acid via an aminotransferase reaction. The oxidation reaction was performed by an yet unidentified enzyme endogenous to *E. coli* (Turk et al., 2015) (Figure 8, purple route). Enzymes for the 2-oxopimelic acid pathway thus exist, but will have to be identified and optimized for improved adipic acid production. Regarding the other four pathways, one or more of the enzymes involved have so far not been proven to exist.

Although the reverse adipate degradation pathway is very similar to the 3-oxoadipic acid pathway, the latter has not been proven to be functional. The main difference between these pathways is the utilization of CoA-bound metabolic intermediates in the reversed adipate degradation pathway, whereas the 3-oxoadipic acid pathway releases coenzyme A from the intermediate, 3-oxoadipyl-CoA, formed in the first enzymatic reaction of the pathway. Also, some of the proven pathways based on fatty acids and glycerol utilize CoA-bound intermediates. Substitution with CoA considerably lowers the K_M values of enzymes utilizing the substituted substrates compared with enzymes utilizing the non-substituted substrates (Bar-Even et al. 2011). Also, enzymes with specificities for CoA-bound metabolites are known and found e.g. in the β-oxidation where fatty acids are broken down to generate acetyl-CoA, NADH and $FADH_2$. The metabolites in the β-oxidation are very similar to the ones in the reverse adipate degradation pathway, apart from that the metabolites in the β-oxidation can contain longer chains of carbon than the metabolites in the reverse adipate degradation pathway. Also, the direction of the reverse adipate degradation pathway is reverse to the β oxidation pathway. Pathways utilizing CoA-unbound metabolites have been less successful, which could partially be due to the fact that they require as yet unidentified enzymes to perform the reaction in question. All in all, these observations could be reasons to why pathways using CoA-bound metabolites for adipic acid production have proven functional whereas pathways utilizing CoA-unbound metabolites have been less successful.

The pathway via lysine

The suggested pathways for the production of adipic acid from glucose described in the literature, both indirect and direct, were briefly reviewed above. In this section, attention is directed to the metabolic pathway investigated in this work, where adipic acid is produced via lysine.

The conversion of lysine to adipic acid via four enzymatic steps has been suggested (Burgard et al., 2012). In the first step, the α-amino group from lysine is removed, yielding 6-aminohex-2-enoic acid (6-AHEA), followed by reduction to yield 6-aminocaproic acid (6ACA). The terminal amino group is then removed, resulting in adipic acid semialdehyde, which is oxidized to adipic acid (Figure 9, pathway I).

The redox potential of the adipic acid pathway via lysine is neutral in terms of NAD(P)H requirement. The NAD(P)H used in the second enzymatic step, the reduction of 6-AHEA to 6-ACA, is regenerated in the fourth enzymatic step, the oxidation of adipate semialdehyde to adipate. The third enzymatic reaction is transamination of 6-ACA into adipic acid semialdehyde. 2-oxoglutaric acid is used in the transamination reaction, yielding L-glutamic acid (Figure 9, pathway I) (Burgard et al., 2012). In the bacterial pathway, 2-oxoglutaric acid is regenerated when aspartic acid is formed from oxaloacetic acid (Averesch et al., 2018). In the yeast pathway, 2-oxoglutaric acid is required both as the starting metabolite for lysine production, and in the third reaction of the pathway. The regeneration of 2-oxoglutaric acid is thus required to avoid the loss of carbon when 2-oxoglutaric acid is converted into L-glutamic acid via the transamination reaction.

The reported high theoretical yield, the NADH/NAD$^+$ balance and the regeneration of 2-oxoglutaric acid in the bacterial pathway make the pathway via lysine interesting. In addition, the bacterium *Corynebacterium glutamicum,* used industrially for lysine production, could potentially be engineered for the production of adipic acid via lysine. *C. glutamicum* has been reported to produce lysine with titers as high as 120 g L^{-1} and a productivity of 4.0 g L^{-1} h^{-1} (Becker et al., 2011; Becker and Wittmann, 2012). However, although the total length of the pathway is rather short, the challenge associated with this pathway is that for three of the four biochemical reactions, there are as yet no identified enzyme capable of performing the desired reaction. Furthermore, it was calculated in the present work that the Gibbs free energy of reaction for two of these biochemical reactions is positive (see table in Figure 9), hence the reaction is thermodynamically unfavorable (**Paper II**). These thermodynamically unfavorable reactions are the deamination of lysine yielding 6-AHEA, and the transamination of 6-ACA yielding adipate semialdehyde (see table in Figure 9). It should be noted that standard conditions were assumed in the thermodynamic calculations. However, the results indicate problematic reactions that should be avoided if possible, by searching for alternative routes and strategies.

Additional pathways via lysine

As pointed out above, the inclusion of as yet unidentified enzymes in three of the four biochemical reactions poses a considerable challenge in the pathway via lysine. The possibility of circumventing this problem by developing an additional pathway was therefore

explored (**Paper II**). Careful data mining from KEGG and BRENDA revealed an additional pathway with fewer biochemical reactions employing as yet unidentified enzymes (Figure 9), pathway II). In addition, no difference was found in the redox balance or the thermodynamics (see table in Figure 9). For simplicity, this pathway will henceforth be referred to as "lysine pathway II" and the original pathway will be referred to as "lysine pathway I".

The higher number of identified enzymes in lysine pathway II is due to the existence of enzymes capable of converting lysine, via the removal of the terminal NH_2 group, to form allysine (Hammer et al., 1991; Hammer and Bode, 1992; Kinzel, Winston, and Bhattacharjee, 1983; Schmidt, Bode, and Birnbaum, 1988). The corresponding reaction in lysine pathway I, removal of the terminal NH_2 group from 6-ACA to form adipic acid semialdehyde has, to the best of the author's knowledge, not yet been observed.

Changing the order of the biochemical reactions in the pathway causes different intermediates to be formed, thereby increasing the number of potential substrates for a certain type of biochemical reaction from one to two (Figure 9). The increased number of potential substrates increases the probability of identifying or engineering enzymes so that they are able to perform a particular biochemical conversion.

To circumvent the first biochemical reaction in lysine pathway I (Figure 9), where there is yet no enzyme identified (deamination of lysine to yield 6-AHEA), a pathway over β-lysine has been proposed (Saez-Jimenez et al., 2019). In this pathway, which is referred to as lysine pathway III, the lysine is converted into β-lysine using lysine 2,3-aminomutase (EC 5.4.3.2), and the β-lysine is then deaminated to 6-AHEA (Figure 9, pathway III).
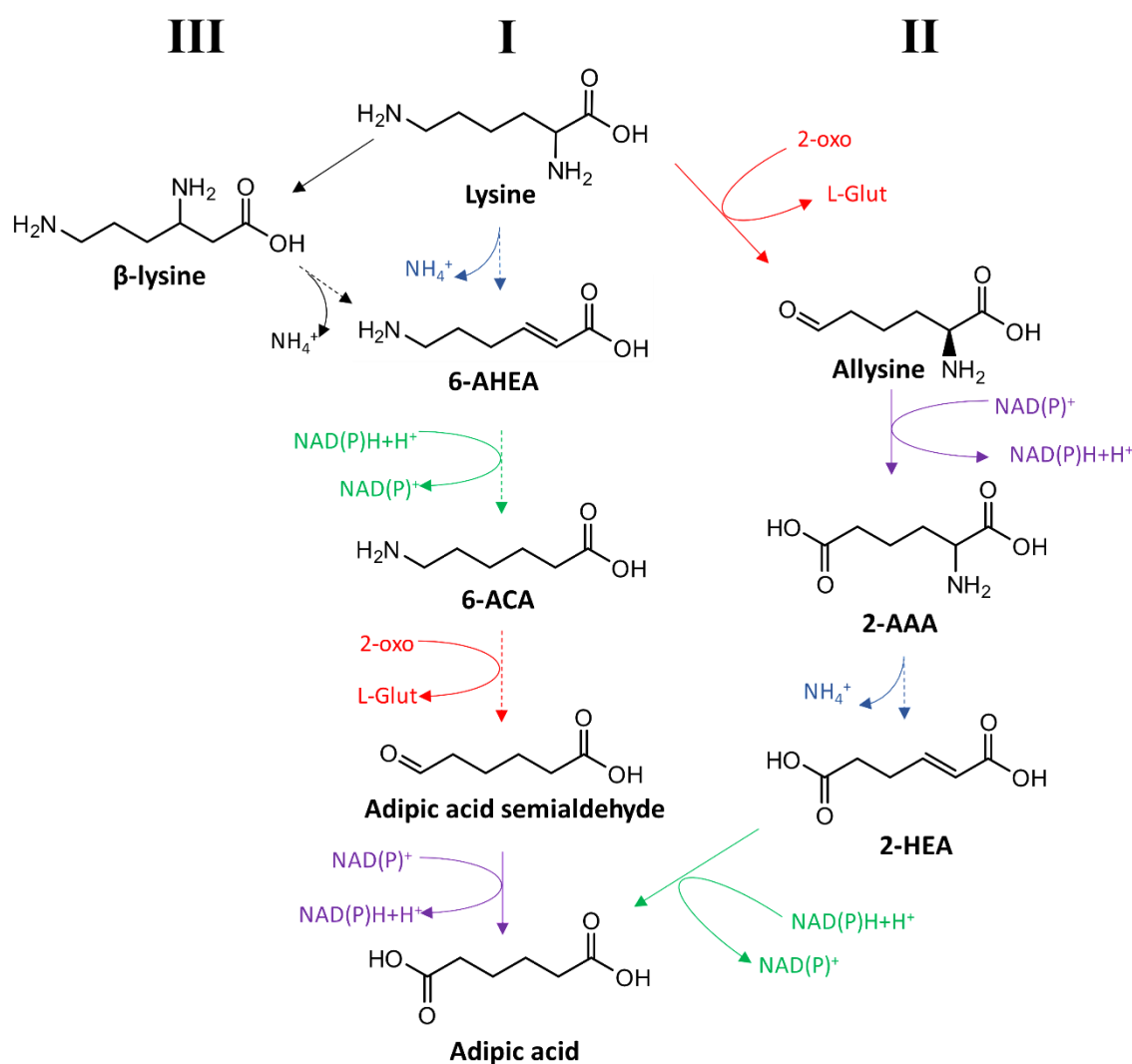
Figure 9. Pathways to adipic acid via lysine. Lysine pathway I as proposed by Burgard et al. (2012), lysine pathway II, as proposed in **Paper II**, and lysine pathway III, as proposed by Saez-Jimenez et al. (2019). Enzymatic activity has been demonstrated for the reactions indicated by full lines, while no proof of natural enzymatic activity has been demonstrated for reactions indicated by dashed lines. The same type of chemical reactions in the lysine pathways I and II are indicated in the same color: blue: deamination of α amino group, green: reduction of double bond, red: removal of terminal amino group, purple: oxidation to carboxylic acid. 2-oxo: 2-oxoglutaric acid, L-Glut: L-glutamic acid, 2-AAA: 2-aminoadipic acid, 2-HEA: 2-hexenedioic acid

3.3 Identification of enzymes

In the three lysine pathways described above, no enzymes have yet been identified for five of the ten possible biochemical reaction steps. In order to realize a metabolic pathway to adipic acid via lysine, the enzymes capable of performing these reactions must be identified. The strategy used in this work, illustrated in Figure 10, relies on manual screening for enzymes capable of performing the desired type of chemical reaction on similar substrates (**Paper II**). Selected enzymes were first tested *in silico* to evaluate their potential to bind and catalyze the desired reaction on the target substrate. Enzymes showing positive results *in silico* were then expressed and purified prior experimentally tested *in vitro*. Enzyme engineering strategies were then proposed based on the *in silico* and *in vitro* results.
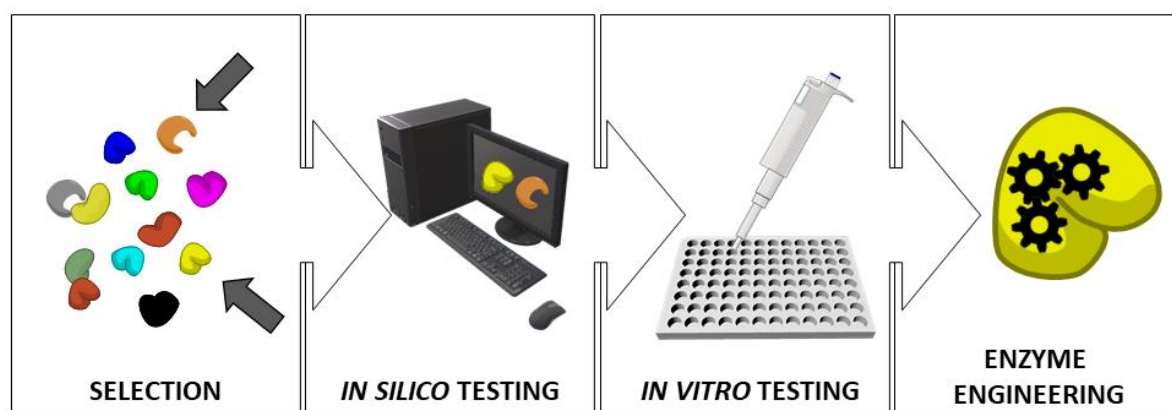
Figure 10. Illustration of the strategy used in **Paper II**: Enzyme selection based on catalytic performance on similar substrates was followed by *in silico* testing to evaluate the potential of the enzymes to perform the desired reaction. Enzymes showing positive results were then tested *in vitro*. Enzyme engineering strategies were then proposed based on the combined results of *in silico* and *in vitro* testing.

Enzyme engineering strategies

Strategies for enzyme engineering can be divided into rational design and directed evolution, although a combination of both can be used (Bilal et al., 2018). In rational design, site-directed mutagenesis is used to alter the amino acid sequence of the enzyme by introducing site-specific point mutations in the gene coding for that enzyme. Detailed knowledge is required of the three-dimensional structure of the enzyme, as well as the reaction mechanisms. This strategy is therefore limited to well-characterized enzymes. However, if no three-dimensional structure is available, homology modeling can be used. In this case, a three-dimensional structure is modeled based on the known three-dimensional structure of an enzyme with high sequence identity to the enzyme of interest. Although rational design has proven powerful (Marshall et al., 2003), the risk of failure is high since our understanding of enzyme function is still incomplete. In fact, mutations far from the active site have been found in evolved enzymes, which could not have been predicted with current tools and knowledge (Arnold, 2018). Directed evolution, on the other hand, mimics natural evolution by the introduction of random mutations and selection of the best performer. Directed evolution generates libraries of enzyme variants via error-prone PCR, DNA shuffling or saturation mutagenesis. Although no structural knowledge of the enzyme is required, an efficient screening method is needed to select for the enzyme with the desired

trait. The application of directed evolution has not only improved existing enzyme properties (Sun et al., 2016), but new, as yet unexplored, enzyme activities have been evolved (Renata, Wang, and Arnold, 2015).

Molecular docking

Experimental screening of enzymes is a tedious and expensive task, since the enzymes must be expressed and purified prior to being tested in an enzymatic assay. Virtual screening, a technique in which computational methods are used instead of experimental methods, can be used to reduce the time and cost of screening. Molecular docking is the most common method for structure-based screening (Meng et al., 2011). This allows interactions between the ligand and an enzyme to be modeled at the atomic level, providing valuable information on the behavior of the ligand in the active site, as well as elucidating fundamental biochemical processes. Thanks to increasing computational capacity, molecular docking methodologies have advanced from the "lock-and-key" theory (where a ligand was regarded as the key and the enzyme as the lock in a rigid structure) (Fischer, 1894; Kuntz et al., 1982), to the "induced fit" theory where the enzyme is continuously reshaped by interactions with the ligand (Hammes, 2002). However, the most popular docking method today involves a flexible ligand and a rigid enzyme, as this is less computationally demanding (Friesner et al., 2004; Moitessier et al., 2008). The different conformations obtained are scored, and the best score ideally represents the experimental binding mode. Although molecular docking can provide valuable information, it is a theoretical model, and a promising docking result does not necessarily mean that catalytic activity will be observed *in vitro* or *in vivo*.

Reduction of the unsaturated α,β bonds of 6-AHEA and 2-hexenedioic acid

In lysine pathways I and II, the enzymes responsible for the reduction of the unsaturated α,β bonds of 6-AHEA and 2-hexenedioic acid (2-HEA) (Figure 11) had until recently not been identified. However, evidence for an enoate reductase capable of converting 2-HEA has now been reported (Joo et al., 2017). The lack of identified enzymes capable of acting on these compounds is probably due to the fact that these compounds are rarely, if ever, found in nature. No hits were obtained when searching for these compounds in the KEGG database.

Enoate reductases are capable of reducing the unsaturated α,β bonds in a range of substrates (Simon and Gunther, 1998; Tischer, Bader, and Simon, 1979). However, enoate reductases are sensitive to oxygen, due to the presence of iron–sulfur clusters; while the lysine pathways require aeration for maximal yield (Burgard et al., 2012). Therefore, enoate reductases were not considered in this study (**Paper II**). The enzyme *N*-ethylmaleimide reductase (NemA) from *E. coli* was chosen instead, since it has been reported to convert 6-AHEA to 6-ACA, although with a very low yield and productivity (<0.5 % mol product per mol substrate after 48 hours of incubation) (Raemakers-Franken et al., 2009). The closely related Old Yellow Enzyme 1 (Oye1) from *Saccharomyces pastorianus* (Warburg and Christian, 1932) was also selected since it has been well studied (Fox and Karplus, 1994), and is known to reduce unsaturated α,β bonds in a broad range of substrates, including aldehydes and ketones (Walton and Stewart, 2013). However, monocarboxylic acids have been reported not to be substrates for Oye1 (Vaz, Chakraborty, and Massey, 1995; Williams and Bruce, 2002),

although a reaction can be obtained when an additional electron withdrawing group, such as a second carboxylate or ester group, a halogen or a nitrile, is added in a position favoring activation of the unsaturated α,β bond (Winkler et al., 2012).
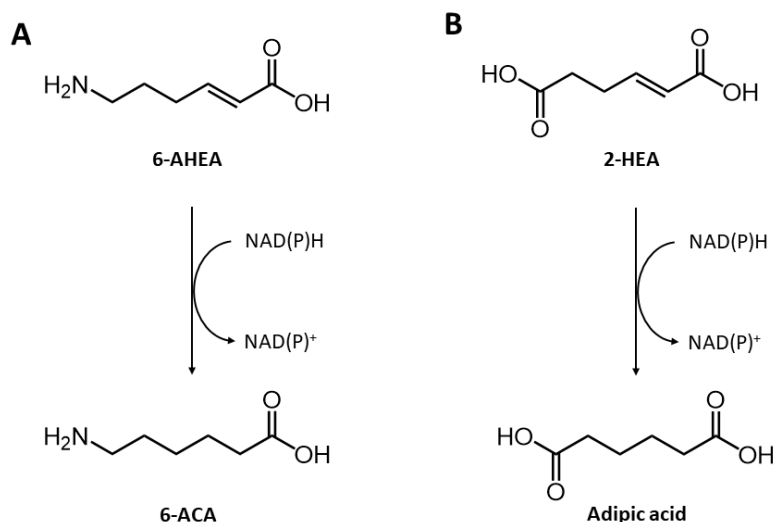
**Figure 11**. Reduction of the unsaturated α,β bonds of: A) 6-AHEA to 6-ACA, and B) 2-HEA to adipic acid.

Docking studies based on the solved three-dimensional structure of Oye1 and a homology model of NemA (the creation of the homology model was part of the work presented in **Paper II**) revealed that both enzymes are promiscuous enough to bind the substrates 6-AHEA and 2-HEA in a position favoring reaction. However, in the case of 2-HEA, the docking results were often in a "flipped" interaction, meaning that the C6 carboxylic group, rather than the preferred C1 carboxylic group, interacted with the enzyme in a position not favoring the reaction (**Paper II**). Based on the positive docking results, experimental testing was performed *in vitro* on both enzymes, however, no activity was observed. For the reaction to occur, the unsaturated α,β bond must be activated. Aldehydes and ketones have electron withdrawing potential which, upon hydrogen bonding with the enzyme, will increase further and activate the unsaturated α,β bond (Figure 12, A). Carboxylic acids, on the other hand, do not have any electron withdrawing potential when deprotonated at neutral pH, and are thus unable to activate the unsaturated α,β bond (Figure 12, B).

It was hypothesized that protonation of the carboxylic acid would induce electron withdrawing potential and favor reaction (Figure 12, C). To test this hypothesis, electron withdrawing potential of the carboxylate group was induced by protonating the acid by lowering the pH. However, the enzymes lost their catalytic activity completely, or more likely were unable to fold properly, at a pH below the pK_a of the target substrates 6-HEA ($pK_a = 4.5$) and 2-HEA ($pK_a = 4.3$). An alternative way of increasing the electron withdrawing potential is by creating additional hydrogen bonds to the oxygen atoms of the carboxylate group. If both oxygen atoms of the carboxylate group form two hydrogen bonds each with the enzyme, this could induce an electron withdrawing potential, and the unsaturated α,β bond would be activated, favoring the reaction (Figure 12, D) (**Paper II**).

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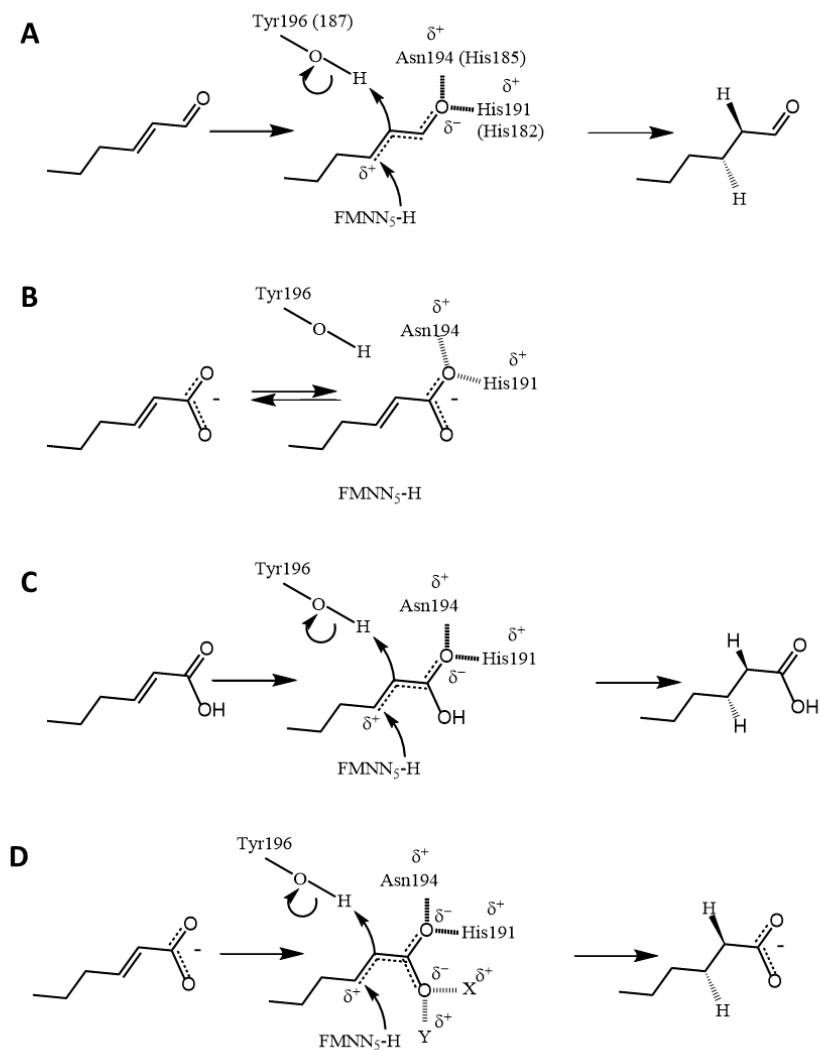
Figure 12. Proposed reaction mechanism of Oye1 and NemA for the reduction of α,β-unsaturated aldehydes, here visualized with *trans*-2-hexenal, and carboxylic acids, here visualized with *trans*-2-hexenoic acid. A) Electrons from the double bond of the aldehyde are shifted towards the catalytic residues Asn194 (His182) and His191 (His185) (dotted lines), thereby creating a partial positive charge on the β-carbon (δ+) of the substrate, which activates the double bond and makes it susceptible for hydrogenation. When the double bond is activated the transfer of a hydride from the flavin N5 to the β-carbon of the substrate and protonation from Tyr196 (Tyr187) can occur, resulting in hexanal as the final product. B) The electrons of the additional negative charge in the deprotonated carboxylic acid are distributed between the two oxygens in a resonance structure (dotted lines). Upon hydrogen bonding with the catalytic residues of the enzyme, the electrons in the resonance structure will prevent activation of the unsaturated α,β bond and no reaction will occur. C) Electrons from the double bond of the protonated carboxylic acid are shifted towards the catalytic residues and reaction can occur as described for aldehydes, resulting in hexanoic acid as the final product. D) Proposed mechanism for the reduction of deprotonated carboxylic acid by engineered Oye1. Engineering of the enzyme by substitution of the native residues with putative X and Y residues could lead to the formation of hydrogen bonds between the enzyme binding pocket and both oxygens of the carboxylate group. Electrons from the double bond are shifted towards the catalytic residues Asn194 and His191 for one of the oxygens, and to the residues X and Y for the other oxygen (dotted lines), thereby creating a partial positive charge on the β-carbon (δ+) favoring reaction, as described for aldehydes. Residues in brackets apply to NemA as visualized in A. The residues of NemA have been omitted in B-D for clarity. Hydrogen bonding is depicted by parallel dashed lines. Movement of electrons involved in the hydride attack and protonation are indicated by curved arrows.

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Deamination of lysine, β-lysine and 2-aminoadipic acid

In lysine pathways I-III, the deamination of the metabolites lysine, 2-aminoadipic acid (2-AAA) and β-lysine (Figure 13, A-C) are biochemical reactions employing as yet unidentified enzymes. In attempts to find or engineer enzymes capable of acting on these substrates, similar approaches to those described in **Paper II** have been used to study 3-methylaspartate ammonia lyase (MAL, EC 4.3.1.2, able to convert 3-methylaspartate to mesaconate) (Saez-Jimenez et al., 2019), and to study aspartate-ammonia lyase (ASP, E.C. 4.3.1.1, able to convert aspartate to fumarate) (D'avino, 2014). MAL and ASP were selected for two reasons: (i) their capacity to perform the desired type of chemical reaction (deamination of an amino group positioned on the α-carbon of a carboxylate group, resulting in a product with an unsaturated α,β bond), and (ii) the similarity of their natural substrates to target substrates (Figure 13, D and E).
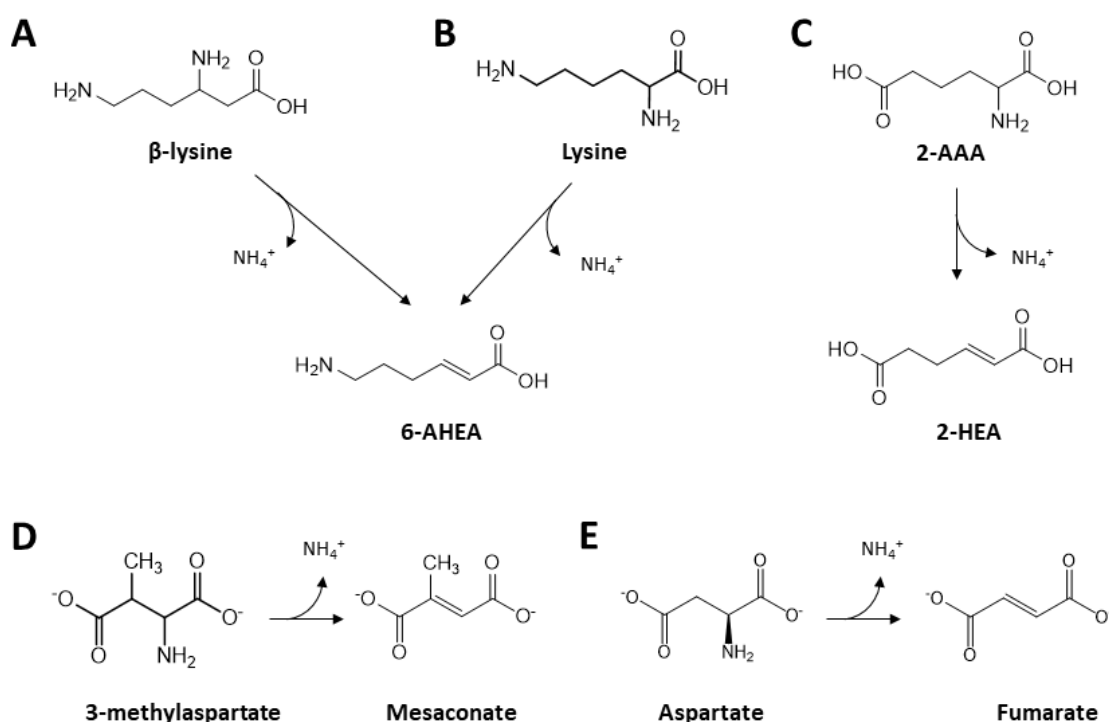
Figure 13. Deamination reactions found in lysine pathways I-III: A) β-lysine to 6-AHEA, B) lysine to 6-AHEA, C) 2-AAA to 2-HEA, D) reaction catalyzed by 3-methylaspartate ammonia lyase and E) reaction catalyzed by aspartate ammonia lyase.

Studies of MAL for deamination of β-lysine, lysine and 2-AAA

In the work by Saez-Jimenez et al. (2019), three different MALs, from *Citrobacter amalonaticus* (CaMAL), *Clostridium tetanomorphum* (CtMAL) and *Carboxydothermus hydrogenoforomans* (ChMAL), were recombinantly produced and tested for activity on lysine, 2-AAA and β-lysine. Despite activity on their natural substrate, 3-methylaspartate (Figure 13, D), unfortunately, no activity was observed on any of the target substrates (Figure **13**, A-C) for any of the enzymes. Instead, β-lysine was found to be a competitive inhibitor of CaMAL, indicating binding to the catalytic pocket. 2-AAA acid was found to be a non-competitive inhibitor, whereas lysine had no affect at all on the enzyme activity. *In*

silico molecular docking studies indicated that the substrates, although more voluminous than the natural substrate, could indeed fit into the catalytic pocket. However, the binding affinities were found to be lower than for the natural substrate. An enzyme engineering approach combining rational design with *in silico* high-throughput saturation mutagenesis was adopted in an attempt to engineer MAL to obtain the desired activity. Target residues deemed suitable for mutation were identified, but unfortunately none of the mutated enzymes showed activity towards any of the target substrates. Despite these negative results, it was concluded that among the target substrates, only β-lysine had a chemical structure suitable as a potential MAL substrate, and that lysine pathway III therefore had the greatest potential for success (assuming activity in succeeding enzymatic reactions).

Studies of ASP for deamination of lysine
In the work by D'avino (2014), two different ASPs, from *E. coli* (AspA) and *Bacillus sp.* YM55-1 (AspB), were recombinantly produced and tested for their activity on lysine. Despite activity on their natural substrate, aspartate (Figure 13, E), no activity was detected on lysine. From *in silico* studies it was suggested that lysine, which is two carbons longer than aspartate, could not fit into the enzymatic pocket due to the tight packing of the active site of the ASPs. Therefore, the substrate binding residues Thr141 in AspB and its equivalent Thr144 in AspA were mutated into glycine via site-directed mutagenesis, to enlarge the substrate pocket. However, the mutated enzymes lost their catalytic activity on aspartate, and no activity was detected on lysine. The folding of the mutant enzymes was not investigated. If proper folding is assumed, the absence of activity on lysine could be due to insufficient hydrogen bonds between lysine and the active site. The additional terminal amino group in lysine could also affect the binding negatively.

Intermediate metabolites of commercial interest in the lysine pathways
The lysine pathways were found to have some intermediates of commercial interest. The identification of such valuable intermediates motivates further research on these pathways. In the best case, both the target product and additional products could result from further studies on the pathway. Such intermediates could serve as an alternative product if the targeted product cannot be produced using one of these pathways, for example, if the enzymes required cannot be identified or engineered.

Intermediate metabolites of commercial interest identified in lysine pathway I were 6-AHEA and 6-ACA, both of which can be chemically converted to caprolactam, a precursor in the nylon industry. The global demand for caprolactam was predicted to reach almost 5 million tonnes in 2017, with a market value of 13 billion USD (Levdikova, 2014), and an expected compound annual growth rate of 3.6 % up to 2023 (Singh, 2019). Caprolactam is currently produced from petrochemical raw materials, and a production route relying on renewable raw materials is therefore desirable. In addition, 6-ACA is FDA approved for pharmaceutical use. The production of 2-HEA via lysine pathway II could be of industrial interest, especially since it is not a drop-in chemical. However, added value is required, for example, in the function of products derived from 2-HEA, compared with the corresponding, existing

petrochemical derived products (Pajalic, personal communication, 2017). Investigations are required on the properties of products derived from 2-HEA to elucidate its potential.

The identification of intermediate metabolites of industrial interest in the lysine pathways is encouraging. The biochemical production of 6-AHEA, 6-AAA and 2-HEA could result in industrially viable processes, even if adipic acid cannot be produced via these pathways. The biobased production of 6-AHEA and 6-ACA via lysine pathway I is of great interest since the identification or development of only one enzyme (conversion of lysine into 6-AHEA) could result in a viable process. Therefore, the deamination of lysine is of significant interest.

4. Selection of a Microbial Host for Production of Adipic Acid

The price of conventionally produced, petrochemically based adipic acid is low (less than 2 USD kg^{-1}) owing to cheap raw materials, a well-established production process, and high production volumes (millions of tons per year). Therefore, it is crucial to keep the overall process cost of biobased adipic acid production as low as possible to ensure economic feasibility. For a biobased process to be competitive with the petrochemical process, the microorganism must be able to produce adipic acid with high titer, productivity and yield. In this chapter, important characteristics of a microbial host for it to be regarded as interesting for industrial adipic acid production will be discussed. Potential microorganisms, based on adipic acid tolerance and low pH process conditions, will be discussed, as well as potential strategies to further improve the tolerance to low pH. Bacteria, yeast and filamentous fungi are considered. Although general considerations are discussed, for example, regarding the effect of acid stress, it is important to note that the physiology of the microorganisms discussed differ in several respects, which calls for different approaches when engineering a microorganism to make it suitable as a microbial host for biobased adipic acid production.

4.1 Desired characteristics of the microbial host

For the production of acids in general, the titer, productivity and yield are required to be in the range 50-100 g L^{-1}, 1-3 g·L^{-1}·h^{-1} and >0.5 g·g^{-1} respectively, to ensure economic feasibility (Huang et al., 2007; Wang, Lin, Xu, Yang, et al., 2016; Warnecke and Gill, 2005). In this section, the characteristics of a microorganism required to achieve an economically feasible biobased process for the production of adipic acid, in particular using the BioBUF concept (see Chapter 2) are outlined. These characteristics are summarized in Box 2, and further explained in the text below.

Box 2. Desired characteristics of a microorganism for economically feasible production of adipic acid using the BioBUF concept

- Well-characterized
- Tolerance to adipic acid, metabolic intermediates and by-products
- Tolerance to lignocellulosic-derived inhibitors in the medium
- Utilization of all sugars present in the medium
- Efficient at low pH process conditions
- Robustness to process fluctuations

In my work, identifying a suitable microbial host for adipic acid production within the setting of the BioBUF concept I focused on tolerance to adipic acid in combination with fermentation at low pH.

Well-characterized microorganism

Since there is no known natural producer of adipic acid, engineering is required to obtain a suitable host with an adipic acid pathway. Engineering a microorganism is substantially easier if its genome has been sequenced and a molecular toolbox is available. In addition, the availability of a genome-scale model is beneficial, as it allows for systems metabolic engineering approaches. A broad spectrum of microorganisms, including bacteria, yeasts and filamentous fungi, are being used in both industry and the research community, and can be considered well-characterized. Among the bacteria, *Escherichia coli* and *Corynebacterium glutamicum* are among the most extensively studied. *E. coli* is often used as a model organism within the life sciences and in industry, and is an important producer of recombinant therapeutic proteins (Huang, Lin, and Yang, 2012). *C. glutamicum* is used in industry for the production of glutamate and lysine, with an annual production of 6 million tons per year (Becker et al., 2018). *Lactococcus lactis* is another well-known bacterium, used in the food industry, where its ability to produce lactic acid from glucose is important in the production of dairy products (Ruggirello, Cocolin, and Dolci, 2016).

Among yeasts, the best-known species by far is *S. cerevisiae*, which has been used throughout history for alcoholic beverage and bread making. Apart from still being widely used in the food industry, it is also used industrially for bioethanol production (Rødsrud, Lersch, and Sjöde, 2012) and for pharmaceuticals e.g. insulin (Nielsen, 2013). Other yeast species used industrially include strains of *Kluyveromyces*, which are used in the dairy industry due to their ability to assimilate lactose (Lane and Morrissey, 2010), and *Yarrowia lipolytica,* known for its ability to grow in hydrophobic environments (Darvishi et al., 2017). Among filamentous fungi, *Aspergillus* species are among those most studied and used in industry. *A. niger* is used for the production of citric acid (Cairns, Nai, and Meyer, 2018) and *A. terreus* for the production of itaconic acid (Wang, Lin, Xu, and Yang, 2016).

*Tolerance to adipic acid, metabolic intermediates and by-products*
High titers are required to reduce the cost of downstream processing, as this can account for more than 50 % of the total process cost (Cheng et al., 2012). To achieve high titers of adipic acid, the microorganism must be able to retain its productivity in the presence of high concentrations of adipic acid.

In addition to tolerance to adipic acid, the microorganism must also have a high tolerance to the metabolic intermediates and the by-products formed. The type of intermediates and by-products formed depends on the metabolic pathway, the microorganism and the process used. Common by-products in the bacterial production of succinic acid are acetate, formate, ethanol and lactate (Song and Lee, 2006), and in organic acid production using *S. cerevisiae* ethanol is an undesirable product (Abbott et al., 2009). When the extracellular concentration of a compound exceeds the intercellular concentration, the concentration gradient will favor diffusion over the cell membrane into the cell. The inflow of an acid into the cell will cause acid stress, which is likely to reduce productivity (Andersson et al., 2009; Guo and Olsson, 2016), and may ultimately cause cell death (Abbott et al., 2009). The mechanisms of acid-related stress are discussed in Section 4.3.

Tolerance to lignocellulose-derived inhibitors in the medium
The types and amounts of inhibitory compounds released depend on the harshness of the pretreatment. Degradation of the sugars may result in the inhibitory furans hydroxymethylfurfural (HMF) and furfural, which can be further degraded into inhibitory weak acids, levulinic acid and formic acid. The inhibitory weak acid, acetic acid, is released from the acetyl groups linked to the hemicellulose polymer, even under relatively mild pretreatment conditions. A diverse mixture of inhibitory phenolics are released from lignin (Almeida et al., 2007). In Figure 14, lignocellulose-derived inhibitors are visualized together with their concentrations found in some lignocellulose-derived streams.

Tolerance of the microorganism to these inhibitors is essential. Detoxification of the medium, reducing the requirements on microbial robustness, is possible via physical, chemical and biological methods (Chandel, da Silva, and Sing, 2011), however, the additional process step will increase the overall cost.
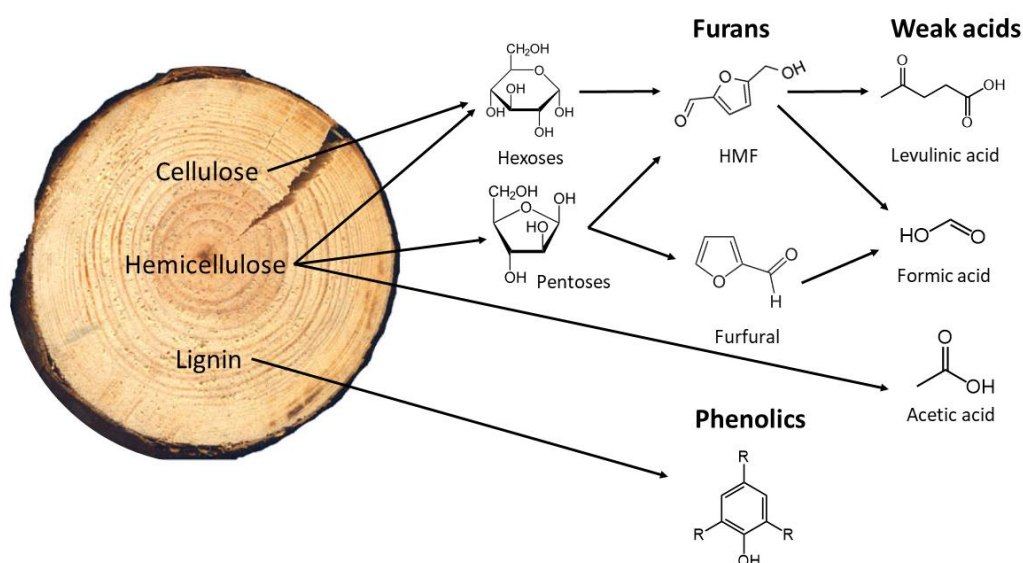
Concentrations (g L^{-1}) of common lignocellulose-derived inhibitors found in various streams of pretreated lignocellulosic raw materials

Raw material	Steam-pretreated Arundo donax[1]	Spruce hydrolysate[2]	Steam-pretreated corncob[3]	Spent sulfite liquor[4, a]
Inhibitor (g L^{-1})				
Acetic acid	0.6-5.7	6.3	8.3-10.4	2.9-7.9
Formic acid	n.r.	1.2	n.r.	1.6-2.8
Levulinic acid	n.r.	2.4	n.r.	0.2-0.5
Furfural	0.1-0.4	1.1	3.8-4.0	0.6-1.2
HMF	0.1	3.4	1.9-2.0	0.1-0.4
Phenolics	n.r.	<0.2	n.r.	1.8-5.1

n.r. – Not reported

[a]Industrial waste stream from spruce pulping

[1]Ask et al. (2012), [2]Koppram, Albers, and Olsson (2012), [3]Koppram et al. (2013) [4]Guo and Olsson (2014a)

Figure 14. Lignocellulose-derived inhibitors and their concentrations found in various streams of pretreated lignocellulosic raw materials.

Utilization of all sugars from the lignocellulosic raw material

Glucose is released from the cellulose after pretreatment and hydrolysis, whereas the hemicellulose contains a mixture of both hexoses (glucose, galactose, mannose) and pentoses (xylose, arabinose). Hexoses are the more abundant sugars, but pentoses can constitute a substantial fraction, depending on the raw material (Table 2). Therefore, the conversion of both hexoses and pentoses is necessary to valorize all the sugars in the raw material.

Table 2. Composition of the various components of lignocellulosic materials as % dry weight excluding ash and extractives (from Olsson and Hahn-Hägerdal, 1996)

Material	Cellulose (%)	Lignin (%)	Hemicellulose (%)	Pentoses (%)
Agricultural waste				
Wheat straw	36	29	28	24
Corn cob	36	-	28	28
Softwood				
Spruce	43	29	26	6
Pine	44	29	26	8
Hardwood				
Birch	40	21	39	25
Willow	37	21	23	14
Aspen	51	16	29	16

Low-pH process conditions

The production of acids will result in a decrease in the pH of the medium unless the pH is adjusted. However, the addition of a base will increase the overall process cost, not only due to the cost of the chemical, but also the cost of its removal in downstream processing. It is also desirable that the product is as pure as possible to facilitate purification, as this will reduce the cost. Adipic acid of high purity is required for the production of nylon as impurities may prevent polymerization (López-Garzón and Straathof, 2014). Furthermore, traditional techniques for the production of acids at near-neutral pH have a negative environmental impact (Jansen and van Gulik, 2014). It is therefore advantageous if the process can be run at acidic pH, and the microorganism must therefore be able to tolerate acidic conditions.

Yeasts such as *S. cerevisiae* and filamentous fungi such as *A. niger* are known to grow at low pH (**Paper III** and **IV**), whereas bacteria such as *E. coli* and *C. glutamicum* prefer neutral pH (Follmann et al., 2009; Presser, Ratkowsky, and Ross, 1997). In fact, low pH can be used as a strategy in bioethanol production to avoid bacterial contamination during fermentation (Kádár et al., 2007). Therefore, yeasts and filamentous fungi are preferable to bacteria when acidic process conditions are required. However, acidic pH conditions will lead to a higher fraction of weak acids derived from the lignocellulosic raw material, e.g. acetic acid and ferulic acid, being present in their undissociated, membrane-diffusible form. Also, the highest titers reported so far for some acids, including the four-carbon-long dicarboxylic acid succinic acid, have been produced by bacteria (López-Garzón and Straathof, 2014; Wang, Lin, Xu, Yang, et al., 2016). This indicates that yeasts are associated with other limitations that must be overcome before *S. cerevisiae* can become an economically feasible host for the production of dicarboxylic acids. Such limitations could be the presence of membrane enclosed compartments in yeast, requiring additional energy for transport of metabolites over the membrane if parts of the metabolic pathway are located inside the compartment. Furthermore, higher titers have been reported at pH 6 than at a pH

below 4 when the filamentous fungus *Rhizopus oryzae* was used for fumaric acid production (López-Garzón and Straathof, 2014). There are also reports of significant inhibition of xylose fermentation by weak acids at low pH (pH 3.5) (Bellissimi et al., 2009). Based on the factors discussed above, it can be concluded that a low process pH could be, but is not necessarily, beneficial for the overall cost of the process. In most cases, the process pH is a compromise between the requirements for fermentation and downstream processing.

Robustness to process fluctuations

Microbial production on the industrial scale is performed in tank reactors. Stirred-tank fermentation and air-lift fermentation are the standard large-scale fermentation processes used in industry (Huang et al., 2007). The type of reactor considered in the BioBUF project was the stirred-tank fermenter. The industrial scale of these fermenters can vary from a few hundred liters to tens of thousands of liters (Laval, 2019). Inadequate mixing and long mixing times are major problems in large-scale industrial, fermenters (Noorman et al., 2014; Schmidt, 2005). Heat transfer may become limiting on the very large scale with decreasing surface area to volume ratio, leading to the need for external cooling (Noorman et al., 2014). Inadequate mixing can lead to the formation of zones with higher stress conditions. The design of reactors used for aerobic processes often involves aeration from the bottom of the tank, resulting in an oxygen gradient, where cells at the top risk to suffer from oxygen limitation, which could affect their metabolism (Baumann et al., 2011; Soini et al., 2008). The addition of an acid, base, antifoaming agent or nutrients usually takes place at the top of the reactor, which may result in inhomogeneity in the fermentation broth. Multiple feeding points can alleviate these problems (Noorman et al., 2014). Given the challenges associated with large-scale industrial fermenters it is clear that the microorganism must be able to cope with variations in the medium during the process. Therefore, robustness of the cell, defined as "persistence of the system's characteristic behavior under perturbations or conditions of uncertainty" (Stelling et al., 2004), is essential if the desired product is to be obtained.

4.2 Potential microorganisms for the production of adipic acid

Selected microorganisms

When selecting a microorganism to engineer for adipic acid production from lignocellulose-derived sugars, the whole process must be considered, including the raw material, the metabolic pathway and the production host. In this work, screening was carried out to find a microorganism with a high tolerance to adipic acid (**Paper III**). The microorganisms *E. coli*, *C. glutamicum*, *S. cerevisiae* and *A. niger* were selected due to their importance in industry and research, the fact that their entire genomes have been sequenced, and that their genetic toolboxes are well-equipped. In addition, genome-scale models have been developed for them allowing for systems metabolic engineering (Brandl et al., 2018; Khodayari and Maranas, 2016; Österlund et al., 2012; Zhang et al., 2017).

The bacterium *C. glutamicum* was also chosen as it is an overproducer of lysine, which is the starting metabolite of the selected metabolic pathway to adipic acid. Both a wild-type

strain and a lysine-over-producing strain of *C. glutamicum* were included in the screening study. The lysine-over-producing strain was included to investigate whether the overproduction of lysine would affect the tolerance of the microorganism to adipic acid. A typical lab strain of *S. cerevisiae* (CEN.PK 113-7D) and an industrial strain (Ethanol Red) were included. Industrial strains of *S. cerevisiae* have a much more complex genetic architecture than laboratory strains, often showing aneuploidy and/or polyploidy rendering them more robust to genetic alterations. Therefore, the results obtained with laboratory strains cannot be extrapolated to industrial strains (Steensels et al., 2014). The two strains of *S. cerevisiae* were chosen to investigate whether tolerance to adipic acid differs in different strains of the same species.

In addition to these well-known microorganisms, two less-known yeast strains, *Zygosaccharomyces bailii* and *Candida viswanathii*, were included in the study. *Z. bailii* is a food-spoilage yeast, and known to be highly resistant to sorbic, benzoic, acetic and propionic acids (Stratford et al., 2013). It was therefore assumed that the tolerance of *Z. bailii* to these acids could also render it tolerant to adipic acid. *C. viswanathii* was included as an engineered strain has been used industrially for adipic acid production from fatty acids, at titers up to 50 g L^{-1} adipic acid (Beardslee and Picataggio, 2012; Picataggio and Beardslee, 2013), indicating that this yeast has a high tolerance to adipic acid.

Tolerance of the selected microorganisms to adipic acid
To investigate the tolerance of the selected microorganisms to adipic acid, their growth was monitored in the presence of increasing concentrations of adipic acid (**Paper III**). The growth of the yeasts and bacteria was monitored in liquid media in a microtiter-plate setup, whereas *A. niger* was grown on a solid medium. Although the results obtained gave good indications of tolerance to adipic acid, submerged growth experiments must be carried out on *A. niger* to better quantify its tolerance to adipic acid.

Increasing concentrations of adipic acid exert increasing osmotic pressure on the cell, which may lead to a reduction in the specific growth rate and cell viability (Mille, Beney, and Gervais, 2005; Rojas, Theriot, and Huang, 2014). To investigate whether an observed decrease in the maximum specific growth rate, μ_{max}, was caused by the effects of adipic acid rather than the change in osmotic pressure, the microorganisms were cultured in a media containing KCl, providing the same osmolality as the medium containing adipic acid. Although the highest osmotic pressures in the control experiments resulted in some reduction in cell growth rate, the effect of adipic acid was greater in all cases, indicating acid- and/or anion-mediated stress (**Paper III**).

The tolerance of the selected microorganisms to adipic acid was compared at pH 6. At this pH, all the microorganisms could grow under the control conditions with no adipic acid present (**Paper III**). The yeasts and the filamentous fungus were found to have substantially higher tolerance to the total adipic acid concentration than the bacterial strains investigated (Figure 15) (**Paper III**). Among the yeasts, *C. viswanathii* was found to be the least affected by adipic acid, showing a decrease in μ_{max} of only 14 ± 4 % at the highest concentration of adipic acid tested (95 g L^{-1}). *C. viswanathii* also showed a higher tolerance to adipic acid

when compared to *S. cerevisiae* in controlled bioreactors (**Paper IV**). Among the bacteria, *E. coli* showed the best tolerance, and could grow at 14 g L^{-1} adipic acid, although μ_{max} was severely reduced (Figure 15). In contrast, the growth of *C. glutamicum* was almost completely inhibited by an adipic acid concentration of only 7 g L^{-1}. These results show that yeasts and filamentous fungi, in particular *C. viswanathii*, have greater potential as host organisms for the production of adipic acid than bacteria.
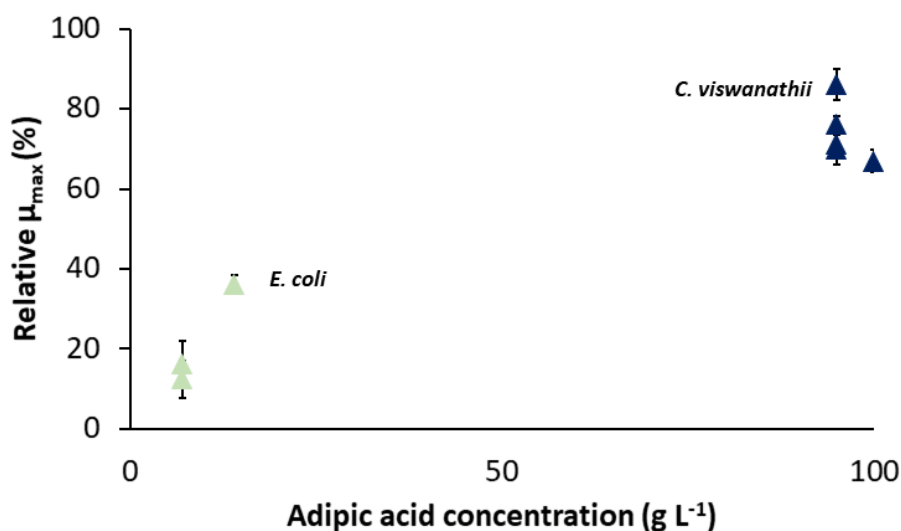
Figure 15. Effect of adipic acid on the μ_{max} for the microorganisms investigated. The results are expressed as relative μ_{max}, corrected for the effect of osmotic pressure, to the μ_{max} of cells grown without adipic acid (%) as a function of adipic acid concentration. The highest concentration of adipic acid at which growth was observed at pH 6 is shown for bacteria (pale green), and for yeast and *A. niger* (dark blue). The highest concentration tested was 95 g L^{-1}, except for *A. niger*, where the highest concentration tested was 100 g L^{-1}. *A. niger* was not cultivated in osmotic conditions hence its growth is not corrected for the effect of osmotic pressure. Each data point is the average ($\pm$ 1 standard deviation) of three to six replicates (**Paper III**).

Other important characteristics of the selected microorganisms
Several characteristics are important, apart from tolerance to adipic acid, in the potential of a microorganism to become a successful adipic acid producer, as presented in Box 2 above. Table 3 presents a summary of these characteristics for the selected microorganisms.

Tolerance to lignocellulose-derived inhibitors is necessary unless detoxification strategies are employed. Studies have been carried out previously on a range of microorganisms, including *S. cerevisiae* and *E. coli*, to determine the inhibitory effects of lignocellulose-derived inhibitors, both as single additions to the medium, and in combination to elucidate possible synergistic effects (Pereira, Verheijen, and Straathof, 2016; van der Pol et al., 2014). Considerable differences have been reported, not only between microbial species, but also between subspecies, making it difficult to draw any general conclusions as to whether one species was more tolerant than another to lignocellulose-derived inhibitors. However, it must be borne in mind that such experiments are often carried out at different pH. *S. cerevisiae* and other yeasts are normally cultivated at a lower pH than *E. coli* and other bacteria, and are thus exposed to a higher fraction of undissociated, membrane-diffusible form of the weak

acids derived from lignocellulose. Thereby, the yeasts are cultivated in a harsher environment compared to bacteria.

Table 3. Summary of the characteristics important for industrial adipic acid production for the selected microorganisms. The level for how well each microorganism relates to each characteristic is indicated from + (low level) to +++ (high level), except for "low-pH cultivation" indicated instead with "Yes" when possible or "No" when not possible.

Microorganism	Well-characterized	Adipic acid tolerance	Low-pH cultivation	Utilization of ligno-cellulosic sugars	Industrially proven
A. niger	+++	+++	Yes	++	+++
S. cerevisiae	+++	+++	Yes	+	+++
Z. bailii	++	+++	Yes	+	+
C. viswanathii	+	+++	Yes	++	+
E. coli	+++	+	No	++	+++
C. glutamicum	+++	+	No	+	+++

Tolerance to lignocellulose-derived inhibitors is necessary unless detoxification strategies are employed. Studies have been carried out previously on a range of microorganisms, including *S. cerevisiae* and *E. coli*, to determine the inhibitory effects of lignocellulose-derived inhibitors, both as single additions to the medium, and in combination to elucidate possible synergistic effects (Pereira, Verheijen, and Straathof, 2016; van der Pol et al., 2014). Considerable differences have been reported, not only between microbial species, but also between subspecies, making it difficult to draw any general conclusions as to whether one species was more tolerant than another to lignocellulose-derived inhibitors. However, it must be borne in mind that such experiments are often carried out at different pH. *S. cerevisiae* and other yeasts are normally cultivated at a lower pH than *E. coli* and other bacteria, and are thus exposed to a higher fraction of undissociated, membrane-diffusible form of the weak acids derived from lignocellulose. Thereby, the yeasts are cultivated in a harsher environment compared to bacteria.

Another important factor is the ability of the microorganism to utilize all the available sugars in the lignocellulosic raw material. The yeasts *S. cerevisiae* and *Z. bailii* are naturally unable to ferment xylose and other pentoses (Subtil and Boles, 2012; Verkleij, 2019), whereas *C. viswanathii* has been reported to co-utilize both hexoses and pentoses from lignocellulosic material (Cao et al., 2017). The filamentous fungus *A. niger* can utilize all the lignocellulose-derived sugars in a sequential manner (Mäkelä et al., 2018). Among the bacteria, *C. glutamicum* is unable to utilize pentoses (Choi, Jeon, and Jeong, 2019), whereas *E. coli* can utilize all the lignocellulosic hexoses and pentoses, although there is a sequential manner in the assimilation of the sugars (Aidelberg et al., 2014; Desai and Rao, 2010).

In addition to being able to utilize all the sugars found in the lignocellulosic raw material, the uptake rate of the sugars is important, since it influences the maximum production rate.

In batch cultivations, the cells grow at μ_{max} during the exponential growth phase, hence their glucose uptake rate is at its maximum. The specific glucose uptake rate during exponential growth of *S. cerevisiae* in minimal medium was roughly twice that of *Z. bailii* (Lindberg et al., 2013), and five times that of *C. viswanathii* (**Paper IV**), demonstrating the ability of *S. cerevisiae* to support a high metabolic flux.

Strains with improved ability to utilize lignocellulosic sugars, as well as improved tolerance to lignocellulosic inhibitors, have been designed. For instance, *S. cerevisiae* has been engineered to utilize xylose, via expression of the XR-XDH pathway, as discussed in Section 3.1. In an alternative pathway, xylose is converted to D-xylulose using a xylose isomerase (XI). Thereby, the difference in cofactor specificities reported for the XR-XDH pathway, leading to production of xylitol and reduced ethanol yield is avoided. Also, the ethanol yield via the XI pathway has been reported to be higher than the XR-XDH pathway (Karhumaa et al., 2007). In addition, long-term adaptation of a xylose-utilizing strain of *S. cerevisiae* in the presence of lignocellulosic inhibitors has led to significantly improved growth in spruce hydrolysate, compared to the parental strain (Koppram, Albers, and Olsson, 2012). The company Genomatica has developed *E. coli* strains capable of co-consuming glucose, xylose and arabinose for the production of 1,4-butanediol, using adaptive evolution and targeted genome editing (Burk, Barton, and Trawick, 2015). Although only a small number of encouraging examples are given here, they demonstrate the possibility of improving future cell factories for the conversion of lignocellulosic biomass into valuable products in an industrial biorefinery.

## 4.3 Inhibitory mechanisms of adipic acid

Acid stress

No reports of acid stress due to adipic acid could be found in the literature. Therefore, more general reports on acid stress were studied. The effect of acid stress depends on the nature of the organic anion (Ullah et al., 2012; Warnecke and Gill, 2005), and the microorganism exposed to acid stress. Here it is important to emphasize the physiological differences between prokaryotes (i.e. bacteria) and eukaryotes (i.e. yeasts and filamentous fungi). While eukaryotes contain compartments surrounded by a lipid membrane, bacteria lack membrane-enclosed organelles. Therefore, enzymatic reactions that take place inside a compartment in a eukaryote, e.g. the TCA cycle in the mitochondria, are protected by a membrane, in contrast to enzymatic reactions in bacteria, which take place unprotected in the cytosol. In addition to their protective compartments, eukaryotes have other physiological differences affecting their tolerance to acids, including differences in the surface-to-volume ratio, membrane lipid composition and internal pH. These, and other differences that may affect their tolerance to acids, are discussed in detail in **Paper III**.

Any acid present in the cytosol will mainly be present in its dissociated form, due to the near-neutral pH of the cytosol. A reduction in the cytosolic pH can cause secondary effects, such as reduced enzyme activity and changes in the electrochemical gradient (Orij, Brul, and Smits, 2011; Warnecke and Gill, 2005). It has been reported that acid stress is mediated by

a dual mechanism, including both pH- and anion-specific stress (Warnecke and Gill, 2005). For instance, the dicarboxylic acid, malonic acid, has previously been reported to inhibit enzymes in the TCA cycle (Pardeet and Potter, 1949). To avoid the negative effects of the acid, the cell must actively remove intracellular acid, either via degradation or export of the acid. When the extracellular concentration of the acid exceeds the intercellular concentration, export of the acid is only possible through active transport at the expense of ATP. If the rate of consumption of ATP exceeds its rate of production, ATP will be redirected from biomass formation to maintain the cytosolic pH close to neutral, thereby decreasing the maximum specific growth rate (Pampulha and Loureiro-Dias, 2000; Warth, 1988) (**Paper IV**). Ultimately, cell growth will be completely inhibited as the acid concentration increases (**Papers III & IV**) (Guo and Olsson, 2014).

Effect of pH on adipic acid

The dicarboxylic adipic acid can exist in three different states: undissociated (H_2A), where both carboxylic groups are protonated, semi-dissociated (HA^-), where one of the carboxylic groups is protonated, and dissociated (A^{2-}), where none of the carboxylic groups are protonated. The equilibrium between the three forms of adipic acid shifts towards the undissociated form as the environmental pH decreases (Figure 16).

	4	5	6	7
H_2A	70.7 %	15.2 %	0.5 %	0.0 %
HA^-	28.2 %	60.6 %	20.0 %	2.5 %
A^{2-}	1.1 %	24.1 %	79.5 %	97.5 %
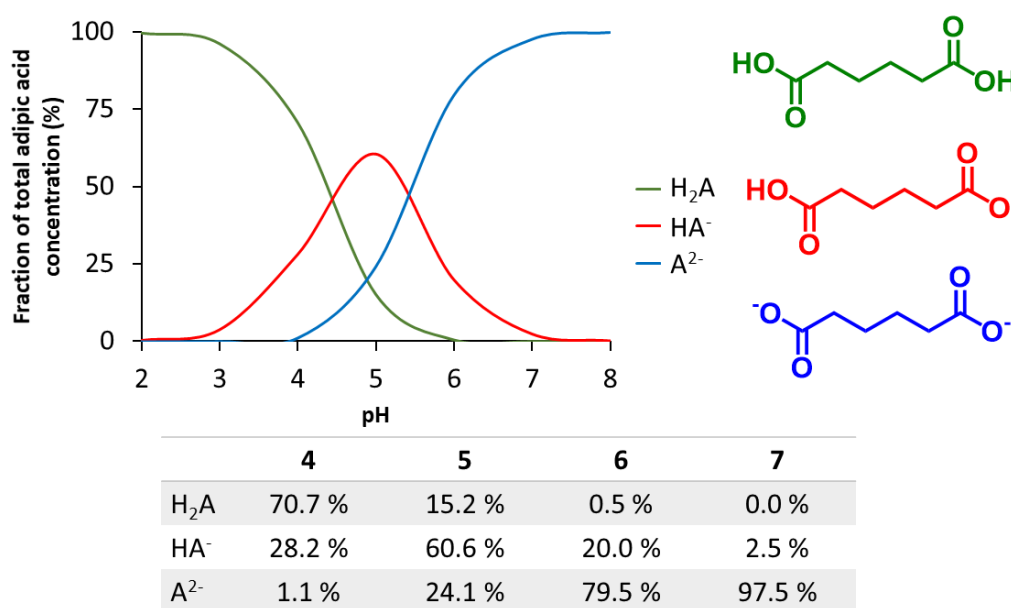
Figure 16. Distribution of the three forms of adipic acid as a function of pH: H_2A - fully undissociated, HA^- - semi-dissociated and A^{2-} - fully dissociated.

Can adipic acid enter the cell via passive diffusion?

To the best of the author's knowledge, no quantitative measurements have been reported on the uptake of adipic acid by any microorganisms. However, small, uncharged, polar molecules can enter the cell via passive diffusion over the plasma membrane when moving from high to low concentrations (Lodish et al., 2000). Experimental evidence for passive diffusion of undissociated, and hence uncharged, dicarboxylic acids over the cell membrane has been reported for both fumaric acid and succinic acid (Jamalzadeh, 2013; Shah et al.,

2016). Considering the structural similarities of these two acids with adipic acid, differing only in two carbon atoms, the entrance of adipic acid, in its undissociated form, into the cell via passive diffusion is likely. This hypothesis is supported by the finding that the lipophilicity of adipic acid (0.2, expressed as log P of the partition coefficient between water and octanol) is between that of the C4 dicarboxylic acids, fumaric (-0.36) and succinic acid (-0.64), and the monocarboxylic acids, benzoic (1.59) and propanoic acid (0.35), which have been reported to enter the cell via passive diffusion (Warth, 1989). Once in the cytosol, the carboxylic groups become deprotonated due to the almost neutral pH in the cytosol, causing acid stress in the cell. Since the equilibrium between the three forms of adipic acid shifts towards the undissociated form as the environmental pH decreases, the cells will experience higher stress with decreasing pH due to increased diffusion of undissociated adipic acid into the cell. Based on the findings of the present work, it is suggested that the semi-dissociated form of adipic acid also plays a part in acid-related stress, due to entrance into the cell via passive diffusion (**Paper IV**).

Effect of undissociated adipic acid on the selected microorganisms
The effect of undissociated adipic acid (H_2A) on the selected microorganisms when grown aerobically on glucose was investigated in this work (**Paper III** and **IV**). The micro-organisms were cultivated in the presence of different amounts of adipic acid and at different pH of the medium, which led to each microorganism being tested over a large range of undissociated adipic acid concentration (**Paper III**). The highest amount of undissociated adipic acid tested for all microorganisms was 5 g L^{-1}. Bacteria were found to tolerate only very low levels of undissociated adipic acid (<1 g L^{-1}), and the value of μ_{max}, in relation to the control conditions, was clearly affected, even at such low concentrations (Figure 17). The yeasts and *A. niger* could tolerate much higher concentrations of undissociated adipic acid, showing a smaller reduction in μ_{max}, in relation to the control conditions, than the bacteria (Figure 17). It was therefore concluded that bacteria are more affected by the presence of membrane diffusible undissociated adipic acid than yeasts and *A. niger*. Remarkably, the yeast strain *C. viswanathii* and the filamentous fungi *A. niger* were unaffected by high levels of undissociated adipic acid when the pH was decreased. *A. niger* was even less affected by increasing amounts of undissociated adipic acid. However, the cultivation method used here was plate growth, and submerged growth experiments must be carried out with *A. niger* to better quantify its tolerance to undissociated adipic acid.
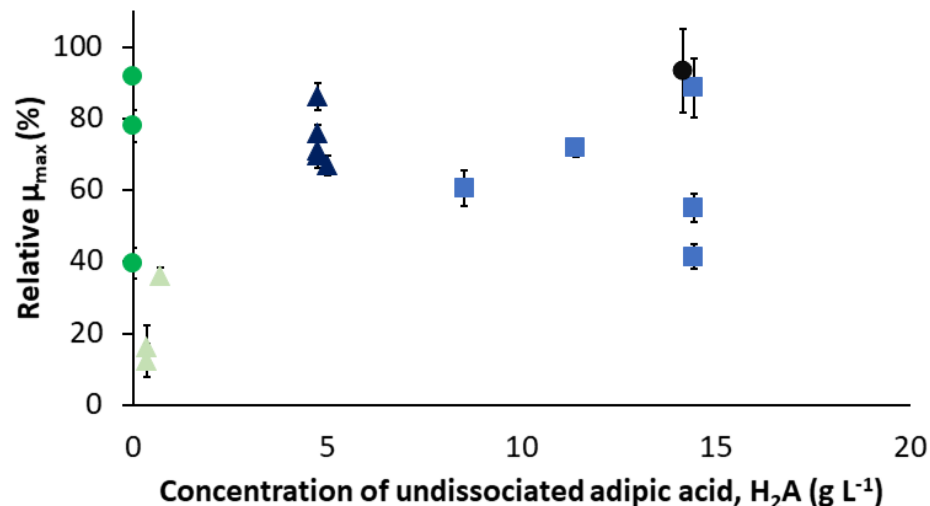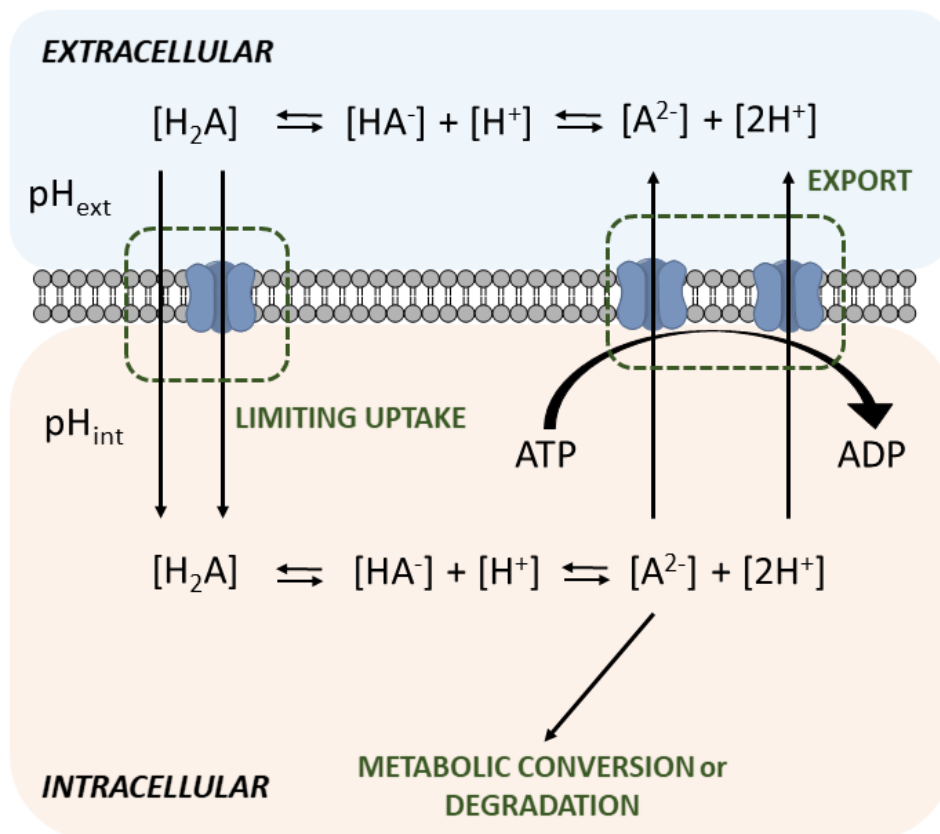
Figure 17. Effect of the undissociated form of adipic acid, H_2A, on the μ_{max} for the microorganisms investigated. The results are expressed as relative μ_{max}, corrected for the effect of osmotic pressure, to the μ_{max} of cells grown without adipic acid (%) as a function of the concentration of H_2A. The highest concentration of H_2A at which growth was observed are shown for *A. niger*, pH 4 (black circle), yeast and *A. niger,* pH 5 (pale blue squares), yeast and *A. niger,* pH 6 (dark blue triangles), bacteria, pH 6 (light green triangles) and bacteria, pH 7 (dark green circles). *A. niger* was not cultivated in osmotic conditions hence its growth is not corrected for the effect of osmotic pressure. Each data point is the average ($\pm$ 1 standard deviation) of three to six replicates (**Paper III**).

Remarks on the results of the screening study

In the screening study (**Paper III**) it was noted that none of the bacteria was able to grow at the highest concentration of adipic acid (95 g L^{-1}), not even at pH 7, where the dissociated form of adipic acid is negligible. The highest concentration of adipic acid at which growth of the bacteria was observed at pH 7 was 56 g L^{-1}. All the bacterial strains investigated could grow at this concentration, although μ_{max} was reduced. The industrial strain of *S. cerevisiae*, Ethanol Red, lost its ability to grow at the highest concentration of adipic acid tested (95 g L^{-1}) when the pH was reduced from 6 to 5. Since the laboratory strain CEN.PK 113-7D could still grow at the highest adipic acid concentration at pH 5, this finding indicates differences in the response to adipic acid within the same species. Interestingly, the food spoilage yeast *Z. bailii,* known to have high tolerance to the monocarboxylic acids sorbic, benzoic, acetic and propionic, was more affected by the dissociated form of adipic acid than the laboratory strain of *S. cerevisiae* and *C. viswanathii*.

4.4 Strategies to reduce adipic acid-related stress

Exposure to high concentrations of adipic acid was shown to reduce or completely prevent the growth of both bacteria and yeast (**Paper III**). Several strategies can be used to reduce the stress related to adipic acid, namely metabolization or degradation of the adipic acid, export of the acid out of the cell, or limiting the uptake of adipic acid (Figure 18). In this section, an overview is given of these strategies, and their potential impact. Due to the lack of experimental results related exclusively to adipic acid, most of the examples given are for other acids exerting a stress on the microorganism.

Figure 18. Overview of the strategies that can be used to reduce adipic acid-related stress of the cells. With reduced extracellular pH (pH_{ext}) the equilibrium of adipic acid is shifted towards the undissociated form (H_2A). The undissociated form of adipic acid (H_2A) can enter the cell via passive diffusion over the cell membrane or via transporters. Upon entrance in the cytosol with nearly neutral pH (pH_{int}), the equilibrium is shifted towards the dissociated form (A^{2-}). The dissociated adipic acid and protons can be exported via active transporter at the expense of ATP. The intracellular adipic acid could also be metabolically converted or degraded.

Metabolic conversion or degradation

Metabolic conversion or degradation of an acid or any type of inhibitor found intracellularly can be used to alleviate the toxic effect. For instance, *S. cerevisiae* cultured in the presence of the lignocellulosic inhibitors coniferyl aldehyde, ferulic acid and *p*-coumaric acid has been found to convert these inhibitors into less toxic products via a series of decarboxylation and oxidation reactions (Adeboye et al., 2015). Combining an NADH-consuming acetate consumption pathway and an NADH-producing xylose utilization pathway for improved ethanol production has also been successfully demonstrated as a means of not only alleviating the toxic effect of acetic acid, but also of utilizing acetic acid for the production of a desired product (Wei et al., 2013). The high acetic acid tolerance of *Z. bailii* has also been attributed to its ability to metabolize acetic acid in the presence of glucose (Rodrigues et al., 2012). Some *Pseudomonas* strains have been found to be able to metabolize or degrade adipic acid (Polen, Spelberg, and Bott, 2013), whereas, to the best of the author's knowledge, the metabolism of adipic acid by yeast has not been reported. Although metabolism can serve as a means of reducing the stress caused by an acid or another type of inhibitor, it cannot be used to reduce the stress caused by the desired product due to reduced yield. Therefore, other means of reducing the stress caused by adipic acid are required.

Export out of the cell

The undissociated form of an acid can be exported out of the cell via passive diffusion over the cell membrane. Passive diffusion is only possible if the concentration gradient favors outflow, i.e., the extracellular concentration must be lower than the intracellular concentration. Assuming a high intracellular concentration of 14.6 g L^{-1} and a near-neutral external pH, titers of less than 2 g L^{-1} were calculated for both *S. cerevisiae* and *E. coli* due to passive diffusion (Averesch et al., 2018). This shows that a high extracellular concentration of adipic acid cannot be reached via passive diffusion, despite a high internal concentration. Higher extracellular titers can only be achieved by active transport over the membrane, at the expense of ATP, especially if the external pH is decreased. Although there are no known active transporters with specificity for adipic acid, the reported titers of adipic acid production by *T. fusca* (Deng and Mao, 2015), *E. coli* (Zhao et al., 2018) and *Candida* species (Beardslee and Picataggio, 2012) indicate the presence of active transporters in these species, rather than passive diffusion. Furthermore, the export of adipic acid eliminates the need for an additional process step to recover intracellular adipic acid.

In the case of dicarboxylic acids, such as adipic acid, the export of the divalent anion requires the export of two protons to maintain charge and pH homeostasis. The ATP required to export dicarboxylic acids is thus higher than for monocarboxylic acids, where only one proton must be exported. Depending on the pH of the external environment, the exported acid may become protonated and will thus be capable of re-entering the cell via passive diffusion. Such futile cycle, where the exported acid will re-enter the cell, will have a huge impact on the net ATP yield, risking decreasing maximum achievable product titers and yields.

Limiting uptake

Avoiding or limiting the uptake of adipic acid into the cell would reduce the ATP required to export adipic acid and maintain pH homeostasis. Limiting the uptake of adipic acid is thus an elegant way of reducing the stress caused by adipic acid since it would save ATP. Uptake of the undissociated form of adipic acid could take place via passive diffusion over the plasma membrane. The presence of transporters with specificity for adipic acid could also lead to the uptake of the acid. Little information is available on specific transporters for adipic acid. The only example that could be found in the literature is the citrate transporter, CitP, in *Lactococcus lactis*. This transporter has been shown to have broad substrate specificity, including the ability to export adipic acid in exchange for the uptake of divalent citrate (Pudlik and Lolkema, 2012). Despite the lack of knowledge on adipic acid-specific transporters, it could be speculated that transporters with specificity for other dicarboxylic acids could also be transporters of adipic acid. In fact, such transporters, including those for succinic and fumaric acids (structurally similar to adipic acid, only differing in length by two carbon atoms) have been reported in both yeast and bacteria (Aliverdieva et al., 2006; Casal et al., 2008, 2016; Jamalzadeh et al., 2011; Youn et al., 2008). The identification of transporters able to transport adipic acid, and their subsequent deletion, could provide a strategy to limit uptake.

In addition to the presence of adipic acid-specific transporters, the membrane composition of the cell can influence the uptake rate via passive diffusion (Berg, Tymoczko, and Stryer, 2002). For instance, it has been suggested that the difference between the cell membranes of *Z. bailii* and *S. cerevisiae* contributes to the improved tolerance of *Z. bailii* to acetic acid (Lindahl et al., 2016). The lipid composition of the cell membrane in *S. cerevisiae* consists of glycerophospholipids (~70%), sphingolipids (~15%) and sterols (15%) (Klose et al., 2012). The presence of sphingolipids and sterols in the cell membrane distinguishes eukaryotes (i.e. yeasts and filamentous fungi) from prokaryotes (i.e. bacteria), since bacteria lack sphingolipids, and only a few bacterial cell membranes have been found to contain sterols (Silhavy, Kahne, and Walker, 2010; Volkman, 2003). Cell membranes are known to adapt to various stresses, including temperature (Klose et al., 2012), salt (Turk et al., 2004) and acetic acid stress (Lindberg et al., 2013). Surprisingly, no major changes were observed in the cell membrane composition of *S. cerevisiae* or *C. viswanathii* when cultivated in the presence of adipic acid (**Paper IV**). However, a clear difference was observed in both total lipid composition and the membrane lipid composition between *S. cerevisiae* and *C. viswanathii* (see Figures 5 and 6 in **Paper IV**). For example, the total chain length of the glycerophospholipids in *C. viswanathii* was longer than in *S. cerevisiae*, indicating a thicker cell membrane. Also, the level of phosphatidylethanolamine was roughly 50 % higher in the cell membrane of *C. viswanathii*, which could indicate a more dense, and thus less permeable, cell membrane than in *S. cerevisiae*. Reduced permeability of acetic acid in *Z. bailii* has previously been shown to be caused by a high content of sphingolipids, resulting in a thicker and more dense cell membrane (Lindahl et al., 2016).

Membrane engineering could serve as a powerful tool to confer improved tolerance to adipic acid on a microbial host by reducing diffusion over the membrane. Targeted approaches, in which the fraction of glycerophospholipids containing acyl chains with 18 carbon atoms was increased at the expense of acyl chains containing 16 carbons, have been successfully applied in *S. cerevisiae* to improve its tolerance to both acetic acid (Zheng et al., 2013) and ethanol (Yazawa et al., 2011). Improved tolerance of *E. coli* to octanoic acid has been achieved by converting the unsaturated fatty acids from *cis* to *trans* conformation (Tan et al., 2016). Although the results of these studies are encouraging, other studies have failed to demonstrate the desired effect. For instance, attempts to increase the sphingolipid content in *S. cerevisiae* resulted in unchanged or even decreased levels of the sphingolipids (Lindahl et al., 2017), indicating that the regulatory mechanisms underlying lipid biosynthesis must be further investigated. Such studies will pave the way for membrane engineering in the development towards cell factories.

5. Conclusions

The conclusions drawn from the research presented in this thesis are presented below. These conclusions provide an important basis for future research with the aim of realizing a microbial cell factory for the production of adipic acid in a biorefinery.

In the study aimed at establishing the **metabolic pathway** from glucose to adipic acid, at least eight different metabolic pathways, with several highly interlinked variants, were identified for both direct and indirect production of adipic acid (**Paper I**). The most common challenges associated with metabolic pathways for the production of adipic acid are redox imbalance, and the presence of biochemical reactions with as yet unidentified enzymes, or enzymes with poor specificity or low catalytic efficiency for the desired reaction. None of the direct pathways identified is superior to the others regarding the maximum theoretical yield, redox balance or number of biochemical reactions employing as yet unidentified enzymes.

The selected pathway, starting with lysine, is redox neutral, but no enzyme capable of performing the desired reaction has yet been identified in three of the four reactions. Database mining was therefore carried out, resulting in the design of an additional pathway, in which the number of biochemical reactions employing as yet unidentified enzymes was reduced without affecting the redox balance or the thermodynamics. This increased the probability of establishing a pathway for adipic acid production via lysine (**Paper II**).

Candidate enzymes for the reduction of the unsaturated α,β bonds of 6-AHEA and 2-HEA (intermediates in the lysine pathways) were NemA and Oye1, capable of reducing unsaturated α,β bonds on substrates with a chemical structure similar to 6-AHEA and 2-HEA. No *in vitro* activity was observed on 6-AHEA or 2-HEA for either NemA or Oye1 (**Paper II**). *In silico* studies revealed that the enzymes NemA and Oye1 could accommodate the substrates 6-AHEA and 2-HEA in their catalytic pockets; thereby increasing the probability of positive results using enzyme engineering (**Paper II**). Enzyme engineering of

NemA and Oye1, aimed at improving the electron withdrawing potential of the carboxylate group, is required for the reduction of the unsaturated α,β bonds of 6-AHEA and 2-HEA.

In the study aimed at identifying a suitable **microorganism** as host for the production of adipic acid from glucose, yeasts and the filamentous fungus *A. niger* were found to have substantially higher tolerance to both total adipic acid and the undissociated, membrane-diffusible form of adipic acid, than the bacteria investigated (**Paper III**). The yeast *C. viswanathii* exhibited the highest tolerance to both total adipic acid and the undissociated form. This indicates that non-conventional microorganisms should be considered in the search for specific physiological characteristics of the microbial host. The superior tolerance of *C. viswanathii* to adipic acid could not be linked to a higher efficiency in generating ATP via respiration than via respiro-fermentation by *S. cerevisiae*. Rather, analysis of the membrane lipid composition revealed that *C. viswanathii* had a thicker and more rigid cell membrane, which is probably less permeable to adipic acid (**Paper IV**).

6. Future perspectives

I envision a future where adipic acid, like most chemicals, is produced from renewable raw materials in a biorefinery. However, this will require significant research efforts in the years to come. This chapter discusses some of the challenges that will have to be overcome, and provides suggestions for future research related to the realization of a microbial producer of adipic acid from glucose.

6.1 A metabolic pathway for the direct production of adipic acid from glucose

To establish a metabolic pathway for the direct production of adipic acid, I suggest three main paths, as outlined below.

Making the pathway via lysine functional
Three pathways to adipic acid via lysine were suggested in this work. None of these pathways has yet proven functional due to the lack of enzymes capable of performing the desired reaction in five of the ten biochemical reactions in these lysine pathways. To identify enzymes capable of performing the targeted reactions, I recommend screening for enzymes capable of performing this type of reaction, i.e. the reduction of unsaturated α,β bonds, on substrates similar to the target substrate. High sensitivity of the screening method is crucial since the efficiency of the desired activity, if present, is likely to be low. In the absence of activity, rational engineering techniques should be employed *in silico* with the aim of identifying strategies to obtain the desired activity. However, this strategy requires the crystal structure of the enzyme to be known, or obtainable via homology modeling. If activity is observed, is it recommended that directed evolutionary engineering strategies be applied to selected enzymes. Large libraries of enzyme variants will be created, requiring robust, high-throughput screening methods.

I recommend continuation of the work on NemA and Oye1 for the reduction of the unsaturated α,β bonds of 2-HEA and 6-AHEA. Directed evolution of NemA should be possible since it has been reported to perform the desired activity on 6-AHEA, although at very low levels (Raemakers-Franken et al., 2009).

I also suggest performing in-depth *in-silico* studies of NemA and Oye1 to identify enzyme engineering strategies to create additional hydrogen bonds to both oxygen atoms of the carboxylate group. It was hypothesized in this work that the creation of such bonds would create the electron withdrawing potential required for the catalytic reaction (**Paper II**). Differences in the electron withdrawing potentials of the catalytic residues should be considered in the engineering strategy adopted. For example, positively charged residues, such as protonated histidine, are more likely to attract electrons and favor the reaction, than polar residues, such as asparagine.

Directed evolution could be explored to develop enoate reductases, which have been reported to reduce 2-HEA to adipic acid, so that they become insensitive to oxygen. In addition, the efficient conversion of 2-HEA to adipic acid is an enzymatic reaction found in four of the metabolic pathways to adipic acid. Development of an enzyme that can efficiently convert 2-HEA into adipic acid will thus improve the probability of realizing one or more of these four pathways.

Development of efficient enoate reductases for the reduction of muconic acid into adipic acid

The indirect pathways via muconic acid were not considered in this work due to the extra cost associated with the final conversion into adipic acid. However, the titers reported for the production of muconic acid from glucose would result in the highest titers of adipic acid reported so far, if the muconic acid was quantitatively converted to adipic acid. In addition, the pathways to muconic acid are reported to have the greatest potential for biobased adipic acid production, based on the yield and titer obtained, compared to the other metabolic pathways, when evaluated *in silico* (Averesch et al., 2018). The recently published reports of direct production of adipic acid via muconic acid in both *S. cerevisiae* (Raj et al., 2018) and *E. coli* (Sun et al., 2018) using enoate reductases, are encouraging. However, the sensitivity of enoate reductases to oxygen could limit their feasibility. The development of efficient enoate reductases for the conversion of muconic acid to adipic acid could pave the way for an efficient metabolic pathway for adipic acid production from glucose.

Overcoming the redox imbalance related to the reverse adipate degradation pathway

Based on the review of all the publicly available metabolic pathways (**Paper I**), the reverse adipate degradation pathway seemed promising, due to its high maximum theoretical yield (>0.90 mol adipic acid per mol glucose) and the fact that it has been functionally proven. However, the reported levels of adipic acid produced from glucose are far from industrially relevant. This could be due to the need for reducing power. The identification of a pathway producing reducing power, linked with the formation of adipic acid, could overcome this problem. I suggest the use of genome-scale models combined with open-minded thinking and brainstorming sessions with others, skilled in the art of metabolic engineering, to

identify novel possibilities. Evaluation of previously used redox engineering strategies are suggested as a starting point. An additional strategy to generate reducing power could be the establishment of a bioelectrochemical system, in which electric power is applied to the bioprocess to generate the reducing power required to balance the reverse adipate degradation pathway. Such systems have been demonstrated to increase yields of a range of products including L-glutamic acid and propionic acid (Rabaey and Rozendal, 2010).

6.2 Developing the cell factory and process for adipic acid production

The non-conventional yeast *C. viswanathii* emerged as a promising microorganism to engineer for adipic acid production owing to its high tolerance to adipic acid, even at low pH (**Papers III** and **IV**). Another example of a non-conventional microorganism with potential for adipic acid production is the bacterium *T. fusca*, reported to possess an endogenous pathway to adipic acid (Deng and Mao, 2015). These two examples demonstrate the benefit of expanding the search for future cell factories beyond conventional industrial microorganisms and embracing the potential of less-known microorganisms. To further explore the potential of *C. viswanathii* and *T. fusca* as industrial producers of adipic acid, their genomes should be sequenced, and molecular toolboxes developed to allow for genetic engineering. The next step would then be to evaluate these microorganisms in industrial settings, using relevant raw materials, for example, pretreated forest residues. The microbial robustness of novel hosts to process fluctuations resulting from scale-up must also be considered when evaluating their potential.

Lignocellulosic raw material was evaluated in the BioBUF project. However, other kinds of raw material could be used, such as more reduced substrates like glycerol or fatty acids. The use of glycerol as substrate resulted in improved adipic acid production compared to glucose via the reversed adipate degradation pathway (Zhao et al., 2018), as well as via reversed β-followed by ω-oxidation (Clomburg et al., 2015), probably as a consequence of improved redox potential in the pathways from glycerol. Crude glycerol is a by-product of biodiesel production, with an estimated production of 17.6 million tons in 2016 (Ahuja and Sonal, 2018). As glycerol is such a cheap and abundant material providing high yields of adipic acid, the use of glycerol may be a better raw material for biobased adipic acid production than glucose. The use of fatty acids as raw materials for adipic acid production using a *Candida* species by the company Verdezyne almost reached commercialization (Wühr, 2011), also demonstrating the potential of using more reduced substrates. Before embarking on a route based on glycerol or fatty acids as raw materials, life-cycle assessments to evaluate the environmental impact for such biorefineries, should be carried out. Sustainability aspects should also be considered, especially in the case of fatty acid production, as this may result in invaluable rain forest being replaced by palm tree plantations.

Realization of a biorefinery for production of adipic acid still requires immense research efforts including development of the cell factory. Here, the knowledge gained in the BioBUF-project serves as a perfect starting point and should be used for continued research.

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